Genetics of Eating Disorders
a candidate gene and a genome-wide association approach

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King's College London

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Genetics of Eating Disorders: a candidate gene and a genome-wide association approach

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Thesis submitted to the University of London for the degree of Doctor of Philosophy (PhD)

Abstract

Eating behaviour is an important aspect of social behaviour, and illnesses like eating disorders thus have a profound impact on quality of life. Family and twin studies provide compelling evidence for the heritability of eating disorders; it is estimated that roughly half of the phenotypic variance is accounted for by genetic factors. The presumed genetic architecture of eating disorders constitutes multiple genetic variants, each with a small effect size. This thesis aimed to replicate findings from the most extensive genetic studies of eating disorders done thus far, in a sample of 700 anorexia nervosa cases and 700 controls from the United Kingdom, the Netherlands, and Austria. The results are non-significant, which is in line with genetic studies of other psychiatric disorders, as well as complex traits such as human intelligence, height, and body mass index. Power to detect genetic risk variants with small effect sizes could be increased by larger samples sizes, and by focussing on disease related quantitative traits rather than diagnoses. Candidate quantitative traits for eating disorders include drive for thinness, bulimia, and body dissatisfaction as measured by the Eating Disorders Inventory (EDI) questionnaire. Chapter 3 of this thesis presents the distribution of these traits in a general population sample from the United Kingdom (n = 3,624 females), and Chapter 4 presents the results of genome-wide association gene (GWAG) analyses of these traits. No gene p values passed a multiple gene testing correction, but among the top genes were several previously implicated in the aetiology of eating disorders. Larger sample sizes would be needed to verify these results. The results of this thesis underscore the phenotypic and aetiologic complexity of eating disorders, but demonstrate that a general population approach using quantitative trait measurements combined with genome-wide hypothesis-free gene analyses can be fruitful.

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Alphabetical list of common abbreviations

AN	anorexia nervosa
B	bulimia - EDI-3 scale
BD	body dissatisfaction - EDI-3 scale
BDNF	brain derived neurotrophic factor
BMI	body mass index
CI	confidence interval
cms	centimeters
Da	Dalton (unit of mass)
ddH20	double distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DT	drive for thinness - EDI-3 scale
DZ	dizygotic
EDNOS	eating disorder not otherwise specified
EDs	eating disorders
FTO	fat mass and obesity associated gene
HWE	hardy weinberg equilibrium
IBD	identity by descent
kb	kilo basepairs DNA
kgs	kilograms
LD	linkage disequilibrium
MAF	minor allele frequency
MZ	monozygotic
OCD	obsessive compulsive disorder
PCR	polymerase chain reaction
QC	quality control
SAP	shrimp alkaline phosphatise
SE	standard error of the mean
SNP	single nucleotide polymorphism
Stdev	standard deviation
UK	united kingdom

Statement of work

Chapter 1

Paragraph 1.3 Genetics of eating disorders was adapted from a review paper written by the author and her supervisor (Helder and Collier, 2011).

Chapter 2

The author coordinated the selection and quality control of the individual phenotype data and DNA samples, and recruited a substantial number of cases for the United Kingdom (UK) case sample from South London and Maudsley National Health Service (NHS) hospitals in London. The other cases from the UK case sample were recruited by the team of Professor Ulrike Schmidt, and Professor Janet Treasure. The control sample from the United Kingdom was part of The Bipolar Association Case-Control study (BACCs) recruited by the team of Professor Peter McGuffin, Professor Anne Farmer, Dr. Georgina Hosang, and Dr. Sarah Cohen-Woods of the Institute of Psychiatry. The case sample from the Netherlands was recruited by the team of Annemarie van Elburg MD, PhD, and Prof. Dr. Roger Adan, from the Rintveld centre for Eating Disorders at Altrecht Mental Health Institute, Utrecht. The Austrian case and control samples were recruited by the team of Professor Andreas Karwautz, Eating Disorders Unit of the Medical University of Vienna. The author conducted the laboratory work, under kind supervision of Rebecca Smith, and performed the statistical analyses, under supervision of Dr. Evangelos Vassos and Professor David Collier.

Chapter 3

Recruitment and data collection was carried out by the UK Adult Twin Registry (TwinsUK). Data quality control and statistical analyses were performed by the author, under supervision of Dr. Evangelos Vassos, Professor David Collier, and Professor Ulrike Schmidt.

Chapter 4

The author participated in an ongoing collaborative project with the Wellcome Trust Sanger Institute in Cambridge, and the UK Adult Twin Registry (TwinsUK). The collaboration included multiple research lines; the author coordinated phenotype data and DNA samples for the London site, and she was involved in the quality control of genome-wide genetic data for the research line on Anorexia Nervosa (Genetic Consortium for Anorexia Nervosa (GCAN). The author was the lead analyst for the genome-wide association gene (GWAG) analyses. The GWAG analyses are post-hoc analyses; Dr. Vesna Boraska (Sanger Institute) was the lead analyst for the GWA single nucleotide polymorphism (SNP) analyses. Since the Anorexia Nervosa research line was behind on schedule due to technical issues regarding genotyping, it was decided for this Chapter to focus on the research line 'behavioural traits related to eating disorders' instead. For this research line recruitment, data collection, and genotyping was conducted by TwinsUK. The results of three different GWAG analyses, analysed by the author under supervision of Dr. Inti Pedroso and Professor David Collier, will be presented in the Chapter. These results are post-hoc to the GWA SNP analyses, which have now been submitted for publication (Vesna Boraska [1,2], Oliver Davis [3], Lynn F Cherkas [4], Sietske G Helder [3], Thomas Pei-Chi Liao [3], Juliette Harris [4], Isabel Krug [3], So-Youn Shin [1], Nicole Soranzo [1], Tim D Spector [4], David A Collier [3], Eleftheria Zeggini [1], *et al*, Genome-wide association analysis of behavioural traits related to eating disorders, *submitted*).

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Acknowledgement of funding

The author was funded by the European Commission (2008-2011) as an Early Stage Researcher from the Research Training Network INTACT (Individually Tailored Stepped Care for Women with Eating Disorders) in the Marie Curie Program (MRTN-CT-2006-035988).

The Genetic Consortium for Anorexia Nervosa (GCAN) enabled the author to visit the Wellcome Trust Sanger Institute in Cambridge for a short period, in order to work on quality control of genome-wide genetic data.

The family foundation Stichting Sint Christophorileen tot Oldehove founded in 1480, Bolsward, Fryslân (The Netherlands), contributed towards the costs of printing and binding the thesis.

Professional acknowledgements

I would like to thank my supervisors David Collier and Ulrike Schmidt; it has been such a great opportunity to work with you and your team, many thanks. Special thanks also go to Evangelos Vassos and Inti Pedroso; thank you for your time and your endless patience in explaining me the statistics. I would also like to thank Vesna Boraska, James Floyd, Chris Franklin, and Eleftheria Zeggini for hosting and supervising me at the Sanger Institute, and for their help with the genome-wide data. Thank you Ricardo Sainz-Fuertes; eventhough proteomics never made it to the thesis it has been a great experience collaborating with you on the mission impossible project. I would also like to take the opportunity to thank Roger Adan for his invaluable support and advice over the years. And lastly a huge thank you to everybody who contributed to the Marie Curie Research Training Network INTACT; it has been a learning experience of a lifetime.

A special acknowledgement is also made to all participants who contributed to this research; this thesis could not have been written without you. Special acknowledgements also to the staff members at the South London and Maudsley National Health Service (SLAM NHS) Foundation trust, specifically from the Eating Disorders Unit and the Phlebotomy department at Maudsley hospital and Bethlem Royal Hospital; thank you for your help with recruitment and data collection, and for your invaluable feedback and support.

Personal acknowledgements

The chances that I am going to forget somebody significant are significant, but here goes… Femke, Miriam, Mieke, Lot, Amy, and Elisabeth; thank you for pulling me through the tough times, endless gratitude goes to you. Family D'Souza and family Pedroso; thank you so much for giving me a safe and loving home when I needed one. Minke, Hayriye, Christelle, Marion, Unna, Myrte, Linda, Kim, Eelke, Christel, Bernard, Rebecca, David D, Sara, Thomas, Isabel, Shaza, Cathy, Inti, Katty, Sarah, Sarah, Bahar, Vangelis, Rudyard, Sabine, Esther, Sanneli, Suzanne, Maria, Fred, Jemma, Mattijs, Ivo, Suus and Syb, Annamieke, Bregtje, Hal, Dorien, Martijn, Paul, Sheena, Pravin, Epco, Marek, and the girls from the King PeaS, everybody from INTACT, from the Eating Disorders Unit, and from the SGDP 2nd floor, and SGDP laboratory; thank you for your contribution, and for making my life so rich. I am dedicating a paragraph to each of you!

Lasty and mostly I would like to thank my family, specifically my parents, and Anton, Karin, Siebren, Nienke, Geert, Ineke, and beppe Sietske; you created the environment in which I could start and finish this thesis. Thank you.

Dedication

This is for my brothers Anthony and Geert,
who are the pillars of my earth.

Introduction

1. Introduction

The everyday act of eating lies at the core of human behaviour. Without food we cannot survive. But food represents much more than basic survival; it is deeply embedded in our cultures as a symbol of comfort and security, of health, nationality, hospitality, social status, and even religion. Illnesses affecting eating behaviour thus have a profound impact on quality of life. The first part of this chapter will discuss the normal regulation of eating behaviour, including hunger and satiety mechanisms, hedonic regulation, and social and cognitive aspects of normal eating behaviour. For disordered eating behaviour three clinical categories currently exist: anorexia nervosa (AN), bulimia nervosa (BN), and eating disorder not otherwise specified (EDNOS). The second part of this chapter will discuss the prevalence and incidence of these eating disorders, their clinical presentation, comorbidity, and risk factors. The third and final part of this chapter will outline the status quo of genetic research on eating disorders. Since genetic studies of eating disorders have typically been non-significant or non-replicated, parallels will be drawn with the genetic architecture of other complex traits, including other psychiatric disorders, human intelligence, height, and body mass index. Understanding the nature of eating behaviour, and specifically the biological processes underlying (aberrant) eating behaviour, will add to the evidence-base needed for intervention in disordered eating behaviour.

1.1. Normal regulation of eating behaviour

1.1.1. Homeostatic regulation

Energy intake and expenditure are carefully matched through the process of energy homeostasis. The gastro-intestinal system and related organs convey the energy status of the body to the brain via hormonal, neural, and metabolic signals upon which energy intake and expenditure are modulated. Leptin is one

of the key proteins involved in energy homeostasis, it is produced by adipose tissue and secreted in direct proportion to body fat mass (Gautron and Elmquist, 2011). Leptin deficiency causes morbid obesity, diabetes, and general neuroendocrine anomalies (Gautron and Elmquist, 2011). Leptin has an important role in one of the earliest systems implicated in energy homeostasis, namely the glucostatic regulation; the glucostatic regulation assumed that changes in plasma glucose regulated food intake (Mayer and Thomas, 1967). Leptin – discovered only in 1994 – affects glucose metabolism through altering insulin sensitivity and hepatic glucose production in an antidiabetic mode (Gautron and Elmquist, 2011).

The most profound and best characterized effect of leptin on the brain is on two neuron populations in the arcuate nucleus of the hypothalamus; pro-opiomelanocortin (POMC) neurons, and neuropeptide-Y (NPY) and agouti-related peptide (AgRP) neurons. Leptin stimulates the anorexigenic POMC neurons and inhibits the orexigenic NPY/AgRP neurons (Cowley et al., 2001). The expression of POMC neurons is regulated by several endogenous molecules, including corticotropin-releasing hormone (CRH), insulin, glucocorticoids (GC), serotonin, and leptin (Shimizu et al., 2007,Heisler et al., 2006). Ghrelin, a well known metabolic protein important in meal initiation, stimulates NPY/AgRP neurons (Briggs and Andrews, 2011). Both neuron populations interact with melanocortin-4-receptor (MC4R) expressing neurons; also key regulators of energy metabolism. MC4R knock-out mice have a metabolic syndrome-like phenotype, and many of the effects of leptin are MC4R dependent (Gautron and Elmquist, 2011). The effects of leptin are however much more wide spread; leptin modulates various aspects of eating behaviour including meal size (Kahler et al., 1998), taste (Shigemura et al., 2004), smell (Julliard et al., 2007), and food reward (Hommel et al., 2006). For example low levels (or absence) of leptin (which normally indicates a state of deprivation) enables mice to find buried food ten times faster than when their leptin levels have been normalized (Getchell et

al., 2006). Meal size for example, which is influenced by gastrointestinal satiation signals including gut peptides like cholecystokinin (CCK) and glucagon-like protein-1 (GLP1) (Zhang et al., 2010), is surveyed by leptin; leptin is able to alter the brain's sensitivity for satiation signals, it overrules short-term homeostasis in order to serve long-term energy homeostasis (Gautron and Elmquist, 2011). Figure 1 gives an overview of the aforementioned homeostatic regulators of eating behaviour.

[image unavailable in e-thesis]

Figure 1: Homeostatic regulators of eating behaviour (Gil-Campos et al., 2006)
The figure shows a selection of the most important homeostatic regulators of eating behaviour (the figure was obtained from reference (Gil-Campos et al., 2006)).

Another key protein implicated in the regulation of energy homeostasis is the brain derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family of growth factors known to stimulate the development and differentiation of new neurons (Alderson et al., 1990,Knusel and Hefti, 1991), as well as promoting long-term potentiation (LTP) (Korte et al., 1996,Patterson et al., 1996); a process of neuronal connection which is key to learning and memory. BDNF is a receptor-ligand with high affinity for the tropomyosin-related kinase B

(TrkB) receptor (Klein et al., 1991,Noble et al., 2011). Lapchak and Hefti noted in 1992 that central administration of BDNF in rats prevented weight gain (Lapchak and Hefti, 1992). Since then many studies have reported effects of BDNF on food intake, body weight, glucose metabolism, physical activity, and metabolic rate (reviewed by (Noble et al., 2011) and by (Rios, 2011)).

In 2007, FTO, another protein important in the regulation of food intake and body weight was discovered (Frayling et al., 2007). This discovery is one of the most prominent successes of non-hypothesis driven genetic research (thus far); Frayling and colleagues found that the protein FTO was associated with type 2 diabetes, and particularly with body mass index (Frayling et al., 2007). Although this protein had first been discovered in 1999 by Peters *et al* (Peters et al., 1999), it was not implicated in the regulation of eating behaviour or body weight until 2007 (Frayling et al., 2007). Funnily enough, in 1999, Peters and colleagues named the gene that they had discovered (through the 'Fused toes (Ft)' mouse mutation): *Fatso* (Fto), inspired by the large size of the new gene (Peters et al., 1999), not knowing this gene would be associated with obesity eight years later. For obvious reasons the gene name has been changed given this new context, it is now refered to as FTO (or: fat mass and obesity associated). Since the FTO discovery, experimental work has focussed on elucidating the biological function of the gene in relation to body weight; experiments in mice have shown that FTO is abundantly expressed in the hypothalamus, and that expression levels are influenced by the nutritional state of the animal (Gerken et al., 2007). There is growing evidence that FTO is associated with increased food intake, and reduced satiety (Day and Loos, 2011).

1.1.2. Hedonic regulation

In times of scarcity survival depends on finding food. Especially during energy depletion food is attributed with reward and pleasure in brain systems to enforce searching. But even in times of plenty energy dense and nutritive food is

extremely rewarding, probably because energy storage is crucial in order to survive famines. In this situation homeostatic signalling must be overruled by the hedonic system. Nowadays prosperous societies have not suffered from famines in decades, but the brain reward system appears unchanged. Energy dense foods are readily available and apparently – given a high prevalence of obesity – homeostatic signalling is unable to prevent overeating. There are two main aspects of the hedonic regulation, for which nomenclature differs between authors, e.g., wanting vs liking; preparatory vs consummatory, appetitive vs consummatory etcetera, but the purpose of all the authors is to distinguish between the phase of wanting and getting the reward versus the phase of actual ingestion. I will refer to this as wanting and liking.

In human eating behaviour these two concepts are very difficult to separate and they cause fierce debates (Havermans, 2011a,Finlayson and Dalton, 2011,Havermans, 2011b), but animal research indicates that two distinct biological processes underlie these concepts. Wanting is driven by incentive expectancies; these are learned expectancies of a hedonic reward (e.g. tasty food, refreshing drinks, sexual partners, addictive drugs etcetera) (Berridge, 2004). The extent of wanting changes equally with the value of the reward. A hedonic incentive is attention grabbing and will elicit goal-directed behaviour (Berridge, 2004), for example: sensing the odour of fresh bread passing a bakery can trigger a sudden urge for food. Dopaminergic projections from the brain's ventral tegmental area (VTA) to the nucleus accumbens (NAc) are most important in this process (Berridge, 2004,Saper et al., 2002). The dopaminergic projections have an effect on wanting, but not on liking. Dopamine depleted mice still prefer sucrose solution over water but initiate licking less frequently (Cannon and Palmiter, 2003). In addition to this, hyperdopaminergic mice run faster in a runway test with sucrose rewards while they do not display a more positive affective reaction to sucrose (Pecina et al., 2003). Liking is the actual sensory pleasure of a reward (e.g. sweet taste) which is unconditioned (Berridge,

2004), and there is strong support for a role of opioids in the liking of food (Barbano and Cador, 2007,Kenny, 2011,Pecina and Smith, 2010). The opioid system regulates food intake by evaluation of the acute rewarding properties of palatable food. Opioid antagonists, such as naloxone or naltrexone, decrease the intake of highly palatable food (Barbano and Cador, 2006), whereas stimulation of opioid receptors enhances behavioural affective reaction and increases food intake (Pecina and Berridge, 2000). For a normal sense of reward both wanting and liking are necessary. If there is no liking then there is no need for wanting, and otherwise a feeling of desire (wanting) complements liking. Imbalanced liking and wanting processes have been suggested to be related to eating disorders and obesity (Finlayson et al., 2011,Berridge et al., 2010).

1.1.3. Social and cognitive aspects of eating behaviour

It is well recognised that there is much more to eating behaviour than just balancing intake and expenditure. Cognitive, environmental, and social factors are, at the least, equally important in the regulation of eating behaviour. Homeostatic signals of a sated state can readily be overridden by learned cues, and equally, cues that signal danger can inhibit feeding even in the food-deprived (Petrovich, 2011). Homeostatic and non-homeostatic signals are so much intertwined that some even argue that a distinction between the two is no longer useful (Zheng and Berthoud, 2007). It has been hypothesized that for the regulation of energy balance it would be too risky to rely solely on changes in homeostatic parameters, rather, most regulation is thought to be anticipatory; to ensure what might be needed is provided for before it is needed, i.e. that the regulation of eating behaviour is aimed at preventing physiological imbalance (Woods and Ramsay, 2007,Benoit et al., 2010). Learning from past experience is of key importance in this, and interestingly the incentive value of certain foods depends on the circumstances; it is assigned cognitively, with higher incentive values assigned to foods when in a state of food deprivation (Benoit et al., 2010,Zheng and Berthoud, 2007). This illustrates the complexity of decision

making in eating behaviour; internal homeostatic signals need to be evaluated in the context of past experience and current environment. Stress and social situations are important aspects of the environment. Perceived psychosocial stress has been linked to eating behaviour and body weight, and stress hormones are known to directly affect food intake (reviewed by (Spencer and Tilbrook, 2011)).

Social norms affect the perception of an individuals own body weight, and are important determinants for changes in eating behaviour (reviewed by Hammond, 2010). Mueller *et al* show that the odds of an overweight girl trying to lose weight depend on whether her peers are also trying to lose weight (Mueller et al., 2010). Social networks actually relate to patterns of obesity; overweight adolescents tend to have overweight friends (Valente et al.,2009). The impact of friendship on eating was tested in a laboratory setting by Salvy and colleagues; they show that more food was consumed when individuals were paired with a friend than when they were paired with an unfamiliar peer, but interestingly – regardless of friendship – overweight youths consumed less food when paired up with a non-overweight peer (Salvy et al.,2009). This social influence on eating behaviour is also apparent when individuals are led to believe that food choices of others were either healthy or unhealthy, people tend to follow the example given to them (Burger et al., 2010). For the genetic studies of eating disorders it is most important to note that genes from a variety of processes (including homeostatic, hedonic, cognitive, and emotional) could harbour genetic risk variants for eating disorders (see Figure 2 for an overview of processes relevant in the normal regulation of eating behaviour).

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Figure 2: Cognitive, reward, and homeostatic regulators (Zheng and Berthoud, 2007)
A variety of processes are important in the regulation of eating behaviour. Apart from homeostatic regulators of energy balance reward, learning, memory, and decision making are important aspects of eating behaviour. This figure illustrates that a multitude of biological processes could harbour genetic risk variants for eating disorders. The figure was obtained from reference (Zheng and Berthoud, 2007).

1.2. Eating disorders

Broadly speaking, eating disorders (EDs) are characterised by distorted beliefs about weight, shape, and eating, and inappropriate behaviour to promote weight loss or to prevent weight gain. They are severe disorders associated with high levels of psychiatric comorbidity, and mortality; AN has the highest mortality rate of any psychiatric disorder (Jacobi et al., 2004b,Hoek, 2006,Swanson et al., 2011,Crow et al., 2009). Furthermore, EDs are associated with an impaired quality of life (Swanson et al., 2011,Jenkins et al., 2011).

Three clinical ED categories are currently recognised in the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV): anorexia nervosa (AN), bulimia nervosa (BN), and eating disorder not otherwise specified (EDNOS). The difference between the categories lies a) in the weight; a diagnosis of AN requires a weight of less than 85% of that expected (or a body mass index (BMI) below 17.5), and b) in the frequency of inappropriate compensatory behaviours (such as self-induced vomiting and binging, i.e. eating an amount of food larger than most people would eat while experiencing a loss of control); which should occur at least twice a week for three months for BN, with all other ED cases categorised as EDNOS (note: the definitions of the categories are currently being reviewed and updated as part of the DSM-V development (http://www.dsm5.org; American Psychiatric Association), for which a series of reviews has been written in the International Journal of Eating Disorders specifically on the categorisation or subtyping of EDs (Peat et al., 2009,van Hoeken D et al., 2009,Wonderlich et al., 2009,Keel and Striegel-Moore, 2009,Striegel-Moore et al., 2009,Becker et al., 2009,Wilson and Sysko, 2009)). In reality it is challenging to 'fit' a patient into these categories, most notably reflected by the disproportionate number of patients in the EDNOS category (Fairburn and Bohn, 2005), and because behaviour is not stable over time (Dalle, 2011,Ackard et al., 2011). Especially the latter is complicating for aetiological research; when patients tend to migrate between categories it becomes very difficult to appoint specific risk factors to the categories, but merging the categories would potentially increase heterogeneity. Generally the weight criterion is considered a hallmark of AN (Bulik et al., 2007a), setting AN cases aside from other EDs. Chapter 2 of this thesis specifically studies the genetics of AN, whereas Chapter 3 and 4 study general ED traits, and for these reasons the next section will focus on general ED aspects, highlighting AN specifically where appropriate.

1.2.1. Prevalence and incidence

Prevalence studies of EDs are relatively recent; before the 1980s there was a general misconception about the frequency of mental disorders, a lack of reliable criteria and diagnostic instruments, and a focus on severe psychotic and neurotic disorders (Jacobi et al., 2004b). The diagnosis BN, as it is known now, was only defined in 1979 by Professor Gerald F.M. Russell from the Institute of Psychiatry in London (Russell, 1979). The general aim of prevalence and incidence (i.e. the rate at which new cases develop) studies is to aid health care management; for health care policy makers it is important to assess the cross-sectional size and scope of mental disorders, and the impairment, help-seeking behaviour, general correlates, and consequences associated with them. Even though these data are of significant value for aetiological research as they may hold important clues about the aetiology (Hoek and van Hoeken, 2003), the aims are however not always in line with the aims of aetiological research. E.g. with respect to aetiology lifetime prevalence of an individual is generally more important than cross-sectional prevalence in a population, and theoretically, there does not have to be any aetiological difference between a patient who receives treatment, and an individual with the disorder never detected by the health care system (Keski-Rahkonen et al., 2007). Community studies show that most EDs are never treated, at least not specifically for their ED (Hoek and van Hoeken, 2003,Keski-Rahkonen et al., 2007,Hudson et al., 2007,Swanson et al., 2011). Hoek and van Hoeken estimate that only one-third of AN cases receives specialised mental health care, and only 6% of BN cases (see Table 1) (Hoek and van Hoeken, 2003).

[table unavailable in e-thesis]

Table 1: Eating disorders at different levels of care (Hoek and van Hoeken, 2003)
Hoek and van Hoeken estimate the one year period prevalence rates in the community

to be 370 for AN and 1,500 for BN per 100,000 young females (Hoek and van Hoeken, 2003). They estimate that only one third of AN cases receives specialised mental health care, and only 6% of BN cases. Most ED cases remain undetected by the health care system.

There are many factors that complicate the interpretation of prevalence and incidence studies, including different diagnostic definitions of disorders (Jacobi et al., 2004b), different definitions of prevalence and incidence (Hoek and van Hoeken, 2003), a learning curve in identification of EDs by primary care (van Son et al., 2006), sampling biases related to urban and rural areas; the prevalence of BN is associated with urban areas, and the same may hold true for EDNOS (Machado et al., 2007), community versus clinical samples (Hoek and van Hoeken, 2003), prevalence and incidence rates differing for different age groups, and for gender (Jacobi et al., 2004b,Currin et al., 2005,Hudson et al., 2007), and differences for birth cohorts, i.e. generation specific differences (Jacobi et al., 2004b,van Son et al., 2006,Hudson et al., 2007). It is very difficult to assess whether there are true increases and decreases in incidence of a disorder, an interesting example of this occurred in the UK in the 1990s: Currin *et al* report an increase in incidence specifically of BN at primary care level, and they speculate that the media attention surrounding Princess Diana's battle with BN may have temporarily decreased the shame associated with the illness, encouraging individuals to seek help for the first time (Currin et al., 2005). The apparent increase in incidence thus may have been caused by a shift in the level of health care utilisation, rather than a true increase of BN.

Generally the lifetime prevalence of strictly defined EDs (by DSM-III and DSM-IV criteria) is 0.3% for AN, and 1% for BN, with higher prevalence rates for females than males (Hoek and van Hoeken, 2003,Jacobi et al., 2004b,Hudson et al., 2007,Machado et al., 2007,Raevuori et al., 2009,Swanson et al., 2011). In Sweden, Finland, and Australia somewhat higher prevalence rates were found for AN in females, up to 2% (Bulik et al., 2006,Keski-Rahkonen et al., 2007,Isomaa et al., 2009,Wade et al., 2006). The peak age of onset of EDs is in adolescence:

roughly between 10 and 20 years of age (Hoek and van Hoeken, 2003,Currin et al., 2005,van Son et al., 2006,Hudson et al., 2007,Keski-Rahkonen et al., 2007,Swanson et al., 2011), though it should be noted that study design can significantly bias age of onset estimates; several researchers note that retrospective age of onset is notoriously unreliable (Jacobi et al., 2004b,Swanson et al., 2011). Despite the fact that most prevalence studies have focussed on Western European and American populations EDs occur cross-culturally, particularly BN and binge eating occur frequently in non-white ethnic populations (reviewed by Hoek (Hoek, 2006))(Swanson et al., 2011).

An important limitation of the prevalence and incidence studies is that they generally focus only on AN and BN, whereas most patients seen in clinical practice are EDNOS (Fairburn and Bohn, 2005). EDNOS includes patients that closely resemble AN or BN, but they fall short on one or more criteria by for example: having a BMI of 18 rather than below 17.5, or because they binge on amounts of food that are not 'objectively' large, or because they do not exhibit the inappropriate compensatory behaviour regularly enough during a period of three months, etcetera. Two specific EDNOS conditions have been described extensively in the literature and are being considered for DSM-V: binge eating disorder (recurring episodes of binge eating) (Wonderlich et al., 2009) and purging disorder (compensatory behaviour for weight and shape control by an individual of normal weight after eating small amounts of food) (Keel and Striegelmoore, 2009). From both a treatment and an aetiological perspective these 'atypical' ED cases are not necessarily different from the currently defined cases (Fairburn and Harrison, 2003). Even though most prevalence studies have only focussed on AN and BN, many studies have included sub-threshold or EDNOS cases, and they show that their prevalence is much higher than strictly defined AN and BN; up to 5% in the community (Hoek and van Hoeken, 2003,Wade et al., 2006,Machado et al., 2007,Keski-Rahkonen et al., 2007,Swanson et al., 2011). It is generally accepted that EDNOS cases are not

necessarily less severe EDs (Schmidt et al., 2008,Machado et al., 2007,Currin et al., 2005,Keski-Rahkonen et al., 2007,Swanson et al., 2011,Fairburn and Cooper, 2011,Dalle, 2011); most notably illustrated by the high mental health services use of sub-threshold ED cases, and the higher number of sub-threshold AN cases with suicide plans and attempts versus strictly defined AN cases found by Swanson *et al* (Swanson et al., 2011). Community studies that have used dimensional measures of disordered eating behaviour have yielded even greater prevalence rates; up to 30% in adolescents in the community (Costarelli et al., 2011,Jones et al., 2001,Treasure et al., 2010). Overall it can be concluded that there is much discussion about the definition of an ED, but it is clear that disordered eating is highly prevalent, especially among female adolescents.

1.2.2. Clinical presentation

Inappropriate eating behaviour usually starts gradually and is often secretive, and can go unnoticed for long periods of time. Friends and teachers may be the first to notice abnormal behaviour and weight loss (Miller and Golden, 2010). Patients may present to primary care with non-specific medical complaints, such as menstrual irregularities, heightened sensitivity to cold, fatigue, constipation, dizziness, or abdominal pain, and their physical appearance may be gaunt and pale, with sunken eyes, and thin limbs (Fairburn and Harrison, 2003,Miller and Golden, 2010). Patients with AN will present particularly with signs of malnutrition and starvation; a (very) low weight, pubertal delay or poor growth, low heart rate and blood pressure, dry skin often with an excess of fine soft hair (i.e. lanugo), scalp hair may be thinning and brittle, and extremities may be cold and cyanotic (Fairburn and Harrison, 2003,Miller and Golden, 2010). There can be many haematological abnormalities including among others anaemia, hypoglycaemia, hypokalaemia, low levels of thyroid hormones, hypercholesterolaemia, and deficiencies in zinc, vitamin D, vitamin K, etcetera (Fairburn and Harrison, 2003,Miller and Golden, 2010). All of these characteristics tend to be secondary to the inappropriate eating behaviour and

weight loss, and are generally reversible with restoration of weight, with the exception of reduced bone density (Fairburn and Harrison, 2003). Osteoporosis is a common and serious consequence of AN, leaving patients with an increased lifetime risk of bone fractures (Mehler et al., 2011). In addition to severe restriction of food intake patients often present with increased physical activity, which contributes to their weight loss (reviewed by (Scheurink et al., 2010)). The core psychopathology of AN is often accompanied by symptoms of depression, anxiety, irritability, impaired concentration, loss of sexual appetite, and obsessionality, all of which typically improve with weight gain (Fairburn and Harrison, 2003).

In patients with BN and EDNOS the physical symptoms are much more subtle; body weight is often in the normal range, though it can fluctuate significantly over time. BN and EDNOS (and sometimes AN) are typically characterised by repeated binges, in which a large amount of food is eaten, accompanied by a sense of lack of control. In most instances the binge is compensated for by self-induced vomiting or misuse of laxatives (i.e. purging) (Fairburn and Harrison, 2003). There can be signs of self-induced vomiting such as enlarged salivary glands, dental enamel erosion, Russell's sign (a thickening of the skin on the back of the hand, caused by the scraping of the teeth when inducing vomiting (Daluiski et al., 1997), and electrolyte disturbances (Fairburn and Harrison, 2003,Miller and Golden, 2010). Typically the behaviour is not regarded as problematic when it is 'successful'; low weight is generally regarded as an achievement, whereas weight gain represents failure (Fairburn and Harrison, 2003). Interestingly in health surveys AN patients tend to report themselves to be in excellent health (Bulik et al., 2006). However overall, binging and purging behaviour is associated with high levels of shame and distress, social withdrawal, and symptoms of depression and anxiety (Troop et al., 2008,Fairburn and Harrison, 2003).

1.2.3. Cross-over and comorbidity

As was mentioned earlier, in reality it is very challenging to 'fit' an individual into a diagnostic category, because there is substantial overlap between the symptoms and because migration between categories over time is very common (Fairburn and Harrison, 2003,Treasure et al., 2010,Dalle, 2011), nicely illustrated by Fairburn (see Figure 3, (Fairburn and Harrison, 2003)). Anderluh *et al* retrospectively assessed lifetime course of EDs in 97 patients, and found that 40% had experienced at least one diagnostic cross-over (Anderluh et al., 2009). Keel and Brown conclude in their review that most AN had transitioned to BN or EDNOS at follow up 2.5 to 18 year later, and that many BN had transitioned to EDNOS (Keel and Brown, 2010). But they also note that AN patients were more likely to retain their AN diagnoses than to fully change into BN, and they speculate that these transitions could represent states of partial remission (Keel and Brown, 2010). A recent community survey of 2,516 adolescents confirms this instability of disordered eating behaviour; 82% of females with self-reported disordered eating at baseline had remained symptomatic five years later, but rarely within the same category (Ackard et al., 2011).

[image unavailable in e-thesis]

Figure 3: Cross-over between diagnostic categories of EDs (Fairburn and Harrison, 2003)
Schematic illustration of the movement between the ED categories, larger arrows indicate the likelihood of direction (Fairburn and Harrison, 2003).

This ‘comorbidity’ within the ED category also holds true for psychiatric disorders in general; most ED patients fulfil at least one set of criteria for an another psychiatric disorder (Jacobi et al., 2004b,Hudson et al., 2007,Swanson et al., 2011,Dalle, 2011,Treasure et al., 2010). In a nationally representative German community health survey it was found that more than 60% of individuals with an ED diagnosis had an additional psychiatric diagnosis (note: ED diagnosis was the 12-month prevalence of ‘any eating disorder’ which included AN, BN, and atypical AN and BN, (Jacobi et al., 2004b)). Jacobi *et al* determined the most frequent combinations of diagnoses, and EDs were not among the five most frequent patterns of comorbidity, unlike depression, anxiety, and somatoform disorders (note: anxiety included obsessive compulsive disorder (OCD), and somatoform included somatisation disorders, hypochondriasis, and pain disorders, (Jacobi et al., 2004b)). This pattern was confirmed by more recent community health surveys; Hudson *et al* found that no single class of disorders stood out as showing frequent comorbidity with ED (Hudson et al., 2007), and Swanson *et al* found that AN was not associated with any particular disorder, apart from ‘oppositional defiant disorder’, and that in contrast, BN was significantly associated with nearly every other disorder (Swanson et al., 2011). In ED research this comorbidity between EDs and other disorders is studied intensively, because it could have significant implications for both treatment and aetiology (Altman and Shankman, 2009,Dellava et al., 2011,Treasure et al., 2010,Dalle, 2011).

1.2.4. Screening for EDs in the general population, and risk factors

It is perhaps not surprising, judging from the previously discussed problematical categorisation of EDs, and the instability of behaviour over time, that screening for EDs in the general population is challenging. It is generally accepted that a structured or semi-structured interview such as the Eating Disorder Examination (EDE) (Fairburn and Cooper, 1993) by a clinician or trained interviewer is the gold standard in diagnosing an ED, but self-report questionnaires are more cost- and

time-effective, especially when assessing a large number of individuals (Jacobi et al., 2004a,Tury et al., 2010). Historically, assessment methods have focussed on early detection of AN (Jacobi et al., 2004a); a complicated undertaking given the low prevalence of stricty defined AN. Many self-report assessment methods have been developed for EDs since (reviewed by (Tury et al., 2010)); some are general measures of disordered eating behaviour (such as the Eating Disorder Inventory (EDI) (Garner et al., 1983)), some are specifically designed as diagnostic instruments (such as the Eating Disorder Examination Questionnaire (EDE-Q) (Fairburn and Beglin, 1994) or the Eating Disorder Diagnostic Scale (EDDS) (Stice et al., 2000)), and some are aimed at screening the general population for individuals at risk (such as the Eating Attitudes Test (EAT) (Garner and Garfinkel, 1979), and the SCOFF (Morgan et al., 2000)) (Tury et al., 2010). The EDI has been labelled as the most comprehensive self-report measure of eating disorders psychopathology, but has been critised for being too long as a screening instrument (Mond et al., 2004,Keski-Rahkonen et al., 2006), and for a lack of validity of the internal factor structure (Garcia-Grau et al., 2010). Because the EDI is the focus of two chapters of this thesis it will be more extensively discussed in the introduction of Chapter 3, page 107.

Several possible risk factors have been identified for EDs, but few have proven to be specific, apart from pregnancy- and birth related complications (Cnattingius et al., 1999,Micali and Treasure, 2009). Generic risk factors associated with EDs include gender, negative self-evaluation, weight and shape concerns, dieting, perfectionism, selflessness, sexual abuse, a family history of EDs, genetics, and general psychiatric morbidity including depression and anxiety (Killen et al., 1994,Fairburn and Harrison, 2003,Jacobi et al., 2004a,Bulik et al., 2006,Dalle, 2011,Jacobi et al., 2011,Bachar et al., 2010). Many of these risk factors overlap with those for other psychiatric disorders, which is not surprising given the high level of comorbidity (also see paragraph 1.2.3 *Cross-over and comorbidity*). From a prevention point of view the specificity of the risk factors is perhaps of

lesser importance, as Stice rightly points out: 'It is arguably more important to identify youth at elevated risk for any eating disorder, because prevention programs should ideally target all eating disorders rather than just one type of eating disorder' (Stice et al., 2011). Stice *et al* found in a eight-year prospective study of 496 adolescent girls that body dissatisfaction was the risk factor with the greatest predictive potency; participants with high body dissatisfaction had a four-fold increase in incidence for disorder onset compared to those with lower body dissatisfaction, and notably among those with high body dissatisfaction, dieting behaviour and symptoms of depression further increased the risk (Stice et al., 2011). Jacobi *et al* found similar interactions of risk factors; in their three-year follow up study in a sample of 236 college-aged women critical comments about eating from teachers or siblings, and symptoms of depression predicted the onset of an ED best (Jacobi et al., 2011).

1.3. Genetics of eating disorders

Genetic risk factors have long been postulated to be important in the aetiology of EDs (Strober and Humphrey, 1987,Holland et al., 1988,Lilenfeld et al., 1998,Strober et al., 2000,Bulik et al., 2000,Klump et al., 2001,Slof-Op 't Landt MC et al., 2005). However, given the complex clinical presentation of EDs (also see paragraph 1.2.2 *Clinical presentation*, page 32) it may be difficult to imagine how genetics could be relevant at all. We have however fewer difficulties accepting that 'something runs in the family', including (very complex) character traits. And we do not object when siblings – born and raised in the same family environment – differ substantially for these (very complex) character traits. Technically speaking, for something to be able to 'run in the family', genetics is likely to be involved, even for very complex traits.

1.3.1. Heritability

In the 1970s genetics was first considered in the aetiology of EDs, because an increased prevalence of EDs was reported in first degree relatives of sufferers

(this history of was described in (Strober and Humphrey, 1987,Rutherford et al., 1993,Gorwood et al., 1998) and (Gordon, 1998)). Correlation between relatives does not prove genetic influences per se, but the results of twin studies also support the notion of a heritable component in the aetiology of EDs (Bulik et al., 2000,Klump et al., 2001,Slof-Op 't Landt MC et al., 2005). Twin studies are based on the difference in correlation of disease between monzygotic (MZ, i.e. identical) twins and dizygotic (DZ, i.e. fraternal) twins, who were born and raised together. The only assumed difference between MZ and DZ twins is their genetic resemblance; MZ twins are considered genetically identical, and DZ twins only share, on average, half of their genes. Genetically DZ twins and regular siblings are equally equal, but DZ twin pairs are a unique 'control group' for MZ twin pairs because they have also shared their prenatal environment, and are of equal age.

The principle of twin studies is based on the following (note: this is a simplification aimed at explaining the principle rather than the details): Theoretically an entirely genetic trait would correlate 100% between MZ twins, and 50% between DZ twins. Any measurement error would lower the percentages, but the correlation between DZ twins would remain approximately half that of MZ twins. Accordingly, a completely environmental trait would correlate equally between MZ and DZ twins, because MZ and DZ twin pairs share their environment to an equal extent, at least, that is the assumption. There has however been much discussion about the assumptions on which twin study designs were based, and not only has the validity of the equal environment assumption been questioned (e.g. (Richardson and Norgate, 2005) and (Mitchell et al., 2007)), the presumed genetical identicality of MZ twins does not hold completely true either (reviewed by (Kato et al., 2005), (Machin, 2009), and (Zwijnenburg et al., 2010)) (note: in this context epigenetic differences are relevant too (Kato et al., 2005,Wong et al., 2010,Campbell et al., 2011)). While genetic discordance between MZ twins is an exciting new opportunity to study

the genetics of disease, it must be noted that the genetic discordance between MZ twins is only minor, and the true effect size of these differences has yet to be established. There is no question that MZ twins have a very much higher genetic resemblance than DZ twins, and even though the heritability estimates may have to be adjusted as they may slightly overestimate the role of genes, they are widely accepted (Manolio et al., 2009,Bulik et al., 2010). As Manolio *et al* point out; the exact heritability estimate is of lesser importance if genetic research leads to one new insight into the aetiology of complex disorders (Manolio et al., 2009), the finding of FTO in obestity is a good example of this (Frayling et al., 2007) (also see paragraph 1.1 Normal regulation of eating behaviour, page 20).

Twin studies of EDs have reported estimated heritabilities of EDs of around 50% (Bulik et al., 2000,Wade et al., 2000,Slof-Op 't Landt MC et al., 2005,Klump et al., 2001,Bulik et al., 2010), with a slightly lower estimate in a recent study using a marginal maximal likelihood approach (Mazzeo et al., 2009). The confidence intervals of these estimates are however generally very wide, e.g. in the 2010 paper by Bulik *et al* the estimated heritability of strictly defined AN was 60%, however the 95% confidence intervals ranged from 0.00 to 0.81 (Bulik et al., 2010). Given the problematic categorisation, the overlap in symptoms between categories, the instability of diagnoses over time, and the generally complex clinical presentation of EDs (see paragraph 1.2 Eating disorders, page 27) this is however not surprising; it is merely a reflection of the complexity of EDs. An important finding from family and twin studies is that EDs do not 'breed true'; i.e. relatives of patients with AN could for example present with BN, or EDNOS (Strober and Humphrey, 1987,Bulik et al., 2007b,Bulik et al., 2010). In 1987 Strober and Humphrey already suggested that the heritability of EDs may lie in their personality traits (Strober and Humphrey, 1987). Personality is a very important concept in EDs (recently reviewed by Lilenfeld (Lilenfeld, 2011)), and several twin studies have studied the heritability of (personality) traits, attitudes,

and temperament in EDs, and have demonstrated substantial heritability (reviewed by Thornton (Thornton et al., 2011)).

1.3.2. Linkage studies

The classical approach to the detection of genes for genetic disorders has been family-based linkage analysis followed by positional cloning. Genetic linkage is the tendency of genetic loci to be inherited together, and family-based linkage analysis aims to associate the inheritance of these loci with the disorder. This approach can be used to find the chromosomal position of disease genes using genetic markers, if a disease gene is close to a particular genetic marker they will tend to be inherited together in a family, or shared by affected relatives. This was very successful for single-gene disorders such as cystic fibrosis, and was consequently attempted for complex disorders such as AN and BN. The difficulties in linkage analysis of complex disorders are likely to relate to their genetic architecture (also see paragraph 1.3.4.3 *Undetected heritability and genetic architecture*, page 52). Large scale genome-wide association studies now show that complex disorders tend to be associated with common, low risk genetic variants (Cichon et al., 2009,Visscher et al., 2011,Day and Loos, 2011). Linkage analysis however is unable to detect these, because the effect size is too small to see in a practical number of pedigrees (Risch and Merikangas, 1996).

Several studies have attempted to detect susceptibility loci for EDs using genome-wide linkage analysis, they have focussed primarily on affected relative pairs with EDs because multiply affected families with AN are very rare. Grice *et al* performed genome-wide linkage analysis of 192 families with at least one affected relative pair with AN and related EDs, including BN (Grice et al., 2002). Analysis resulted in modest evidence for linkage at marker D4S2367 on chromosome 4, and when they reduced the sample heterogeneity by only including families with at least two relatives with restricting AN (n=37) they

found suggestive evidence for an AN-susceptibility locus on chromosome 1 (Grice et al., 2002).

Since AN is an unstable diagnosis, studies have also used quantitative traits for linkage, including those related to other psychiatric disorders, personality, and temperament in eating disorders. Devlin *et al* used two quantitative traits, drive for thinness (DT) from the Eating Disorders Inventory (EDI), and obsessionality from the Yale-Brown Obsessive Compulsive Scale (Y-BOCS), in 196 multiplex families with an AN proband (note: the same sample as Grice *et al* (Grice et al., 2002)) for genome-wide quantitative trait linkage (Devlin et al., 2002). Using a novel method that incorporates covariates, they found several regions of suggestive linkage: one close to genome-wide significance on chromosome 1 for a combined drive-for-thinness–obessionality phenotype, another on chromosome 2 for obessionality only, and a third region on chromosome 13 for drive for thinness (DT) only (Devlin et al., 2002).

Linkage studies have also been used to examine BN (Bulik et al., 2003), using microsatellite markers in 308 multiplex families with EDs identified through a proband with BN; the highest nonparametric multipoint maximum LOD score (MLS) was 2.92, on chromosome 10p. A symptom marker of BN, self-induced vomiting, was also used as a linkage phenotype in a subset of families with at least two affected relatives reporting this, which produced the highest MLS (3.39) at the same locus on chromosome 10p, indicating this region may harbour susceptibility alleles for BN. Interestingly, this region on chromosome 10 was also associated with obesity (Hager et al., 1998). As patients with BN pre-morbidly tend to have a higher BMI, the chromosomal region might point to a gene relevant for both body weight regulation and eating disorders. Linkage analyses of behavioural phenotypes related to EDs, such as obsessionality, age at menarche, anxiety, lifetime minimum body mass index (BMI), concern over

mistakes, and food-related obsessions have also been conducted (Bacanu et al., 2005). Significant linkage was found for minimum BMI at chromosome 4 (4q21.1), for concern over mistakes at chromosome 16 (16p13.3) and chromosome 14 (14q21.1), and for food-related obsessions (14q21.1) (Bacanu et al., 2005). No significant loci were found for AN and overlap between the AN and BN cohorts was minimal for substantial linkage signals (Bacanu et al., 2005).

1.3.3. Candidate gene studies

The candidate gene approach examines genes which are suspected of being involved in a disease, because the function of the gene product suggests that it could be related to the pathophysiology of the disease. For example, in type 2 diabetes (T2D), two candidate genes from the insulin pathway have been successfully examined for association with the disease (Prokopenko et al., 2008). The main flaw of candidate gene studies is the low prior probability of association, since for most diseases, the task of selecting the correct candidate gene from the 30,000 or so human genes is very difficult, even when there is detailed knowledge of pathophysiology, since the function of most genes is poorly characterised. Because of this, but also because of other issues such as statistical power and poor genetic coverage, candidate gene studies have had limited success (Tabor et al., 2002,Kim et al., 2011). For example, despite hundreds of candidate gene studies in T2D, only two have stood the test of time: the Pro12Ala variant in the PPARG gene, involved in insulin action, and the Glu23Lys variant in KCNJ11 gene, involved in β-cell dysfunction (Prokopenko et al., 2008).

In psychiatric disorders candidate gene studies are even more difficult as there is even less information on pathophysiology, thus it is more difficult to select good candidate genes. However there have been some successes: attention deficit hyperactivity disorder (ADHD), which is commonly treated by dopaminergic system drugs such as methylphenidate, has been associated with genes from the

dopamine system, particularly the dopamine D4 and D5 receptor genes DRD4 and DRD5 (Thapar et al., 2007). There has been a series of candidate gene studies in EDs, which examined neurotransmitter or other pathways related to behaviour, such as the serotonin and dopamine systems, the neuropeptides implicated in eating behaviour (also see paragraph 1.1 Normal regulation of eating behaviour, page 20), or genes associated with obesity (Pinheiro et al., 2010,Rask-Andersen et al., 2010b,Scherag et al., 2010). Few if any of these candidate-gene association studies have replicated genetic association. Some findings remain interesting if controversial, in particular agouti-related protein (AgRP) (Vink et al., 2001), brain derived neurotrophic factor (BDNF) (Ribases et al., 2003,Ribases et al., 2004), and the serotonin, dopamine, and opioid receptor types (Collier et al., 1997,Gorwood et al., 2002,Ziegler et al., 1999,Bergen et al., 2003,Brown et al., 2007,Kiezebrink et al., 2010,Bergen et al., 2005,Dmitrzak-Weglarz et al., 2007).

Brandys *et al* examined several genetic variants associated with body mass index (BMI), identified by genome-wide studies of obesity, for association with AN, in a sample of 267 AN patients and 1,636 population controls (Brandys et al., 2010). They found no evidence that genetic variants regulating BMI in the general population are significantly associated with susceptibility to AN. One variant not included in these analyses was the Val66Met variant in the BDNF protein. However in a later meta-analysis Brandys *et al* tested the evidence for association with this variant specifically; this functional single nucleotide polymorphism (SNP) (also identified as rs6265), is a missense variation; the two different alleles of the BDNF Val66Met variant (note: 66 refers to the location of the variant in the protein), guanine (G) and adenine (A), lead to two different amino acids, Valine (Val) and Methionine (Met), and they lead to a different protein structure of BDNF (Noble et al., 2011). This variant is a strong candidate in the aetiology of EDs; in obesity, genome-wide association (GWA) studies found significant association, with the Val66 allele increasing the likelihood of higher

BMI (Thorleifsson et al., 2009). Meta-analyses by Brandys *et al* in a sample of 2,767 AN cases and 3,322 controls were non-significant for association with AN, and the authors concluded that the BDNF Val66Met is not associated with AN, at least not at (currently) detectable levels (Brandys et al., 2011).

The most extensive candidate gene study of AN done thus far studied over 5,000 SNPs in 182 genes using a sample of 1,085 AN cases and 677 controls (Pinheiro et al., 2010). Pinheiro *et al* selected the SNPs on the basis of previous association data, gene expression in the brain, biological plausibility, and markers from regions linked to AN in family based linkage studies (Pinheiro et al., 2010). After accounting for multiple testing, there were however no statistically significant associations (Pinheiro et al., 2010). The authors state that their results nevertheless include a number of potentially interesting findings, including the receptor of the proglucagon-derived gut peptide (GLP2R), the phenylalanine hydroxylase (PAH) gene, and a small-conductance calcium-activated potassium channel (KCNN3), expressed in the brain. For KCNN3, a repeat polymorphism in the gene has previously been associated with AN (Koronyo-Hamaoui et al., 2004,Koronyo-Hamaoui et al., 2002). Larger samples will however be required to verify these results.

1.3.4. Genome-wide association (GWA) studies

Since these studies, advances in genotyping technologies have allowed the development of several genome-wide association (GWA) studies; these studies analyse hundreds of thousands genetic variations at once, and do not rely on assumptions, or hypotheses, on the underlying aetiology of a disorder (Corvin et al., 2010). Two very important concepts in GWA studies are linkage disequilibrum (LD) and multiple testing, because they are very important for the interpretation of the results of this thesis, this paragraph will start with a detailed explanation on them, followed by an overview of results achieved thus far through use of this relatively new genome-wide genetic association method. Because only one small

scale GWA study on AN has been published thus far (Wang et al., 2011), which found no significant results after correction for multiple testing, parallels will be drawn with other complex disorders and traits, such as other psychiatric disorders, body mass index, human intelligence, and height.

1.3.4.1. Linkage disequilibrium, and multiple testing

Genetic variants in the DNA are not independent of each other, especially when they are located closely together (in the DNA) they tend to be inherited together (in their review on the genetics of height, McEvoy and Visscher elegantly discuss the basics of genetic variants and inheritance (McEvoy and Visscher, 2009)). One paternal and one maternal copy of the DNA are transmitted to the offspring via so called 'gametes' i.e. the sperm and the egg cell. During the generation of the gametes the DNA 'recombines', through the process of 'crossover'. This recombination generates a new unique combination of DNA (Lichten et al., 2011). In the human genome, there are two to three crossover events between each pair of chromosomes in the formation of gametes. There are obvious constraints to this process, because genomic integrity needs to be maintained; aberrant crossovers would have deleterious outcomes, with severely problematic recombination being lethal for the embryo, and less severely problematic recombination being associated with developmental disabilities and disease (Coop and Przeworski, 2007). Recombination is not completely at random, there are so-called 'recombination hotspots' (i.e. 'exchange sites') in the DNA, it has been estimated that there are approximately 25,000 such hotpots in the human DNA (Coop and Przeworski, 2007).

This process of recombination and crossover causes the chromosomes to be shuffled into new combinations. Over the course of many generations, as crossover events accumulate, chromsomes become increasingly different between families; divergence increases over multiple generations, and between populations and ethnicities. Within families, (sub)populations, and ethnicities,

there is however significant overlap of genomic regions; the more closely related individuals are, the more equal their DNA is. This relatedness can be very problematic for genetic research; if controls are not sampled from exactly the same population as the cases, it can cause spurious genetic associations. In 2003 the International HapMap project (HapMap, 2003) set out to map common patterns of genetic variation in the population by genotyping the DNA of individuals from various ethnicities. They calculated the pairwise correlation between any two genetic loci (i.e. DNA base pairs; I refer to these as 'genetic variants') in a given population; these data can be looked up e.g. via their online genome browser: http://hapmap.ncbi.nlm.nih.gov or through software tools such as Haploview (Barrett et al., 2005).

When two genetic variants occur together in the genome more often than expected by chance we say they are in 'linkage disequilibrium (LD)', i.e. they are correlated in the given population; when one genetic variant is found in an individual (from the given population) it is very likely the correlated genetic variant will be present in this individual as well. Strongly correlated genetic variants are said to be in 'strong LD', and when genetic variants do not occur together more often than expected by chance they are said 'not to be in LD'. Short genomic regions that are in strong LD are referred to as haplotype blocks; they are comprised of genetic variants in strong pairwise LD, i.e. haplotypes are 'blocks' of DNA inherited together across many generations (Browning and Browning, 2011). Since haplotypes are the focus of Chapter 2 of this thesis they will be discussed in more detail in the next chapter (paragraph 2.1 Literature background, page 59). Figure 4 and Figure 5 illustrate recombination processes, and linkage disequilibrium.

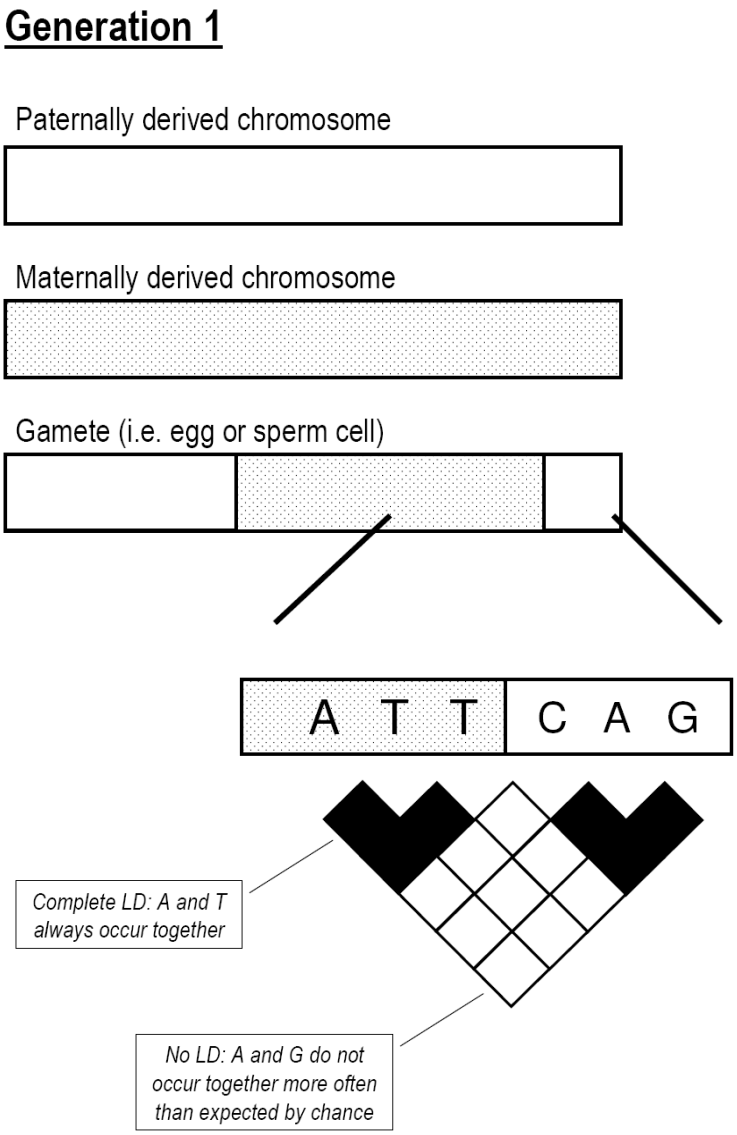

Figure 4: The principle of linkage disequilibrium - generation 1
In gamete formation the chromosomes are 'shuffeled' into new combinations through a process of recombination and crossover. Over the course of many generations, as crossover events accumulate, chromsomes become increasingly different between families (also see Figure 5). Linkage disequilibrium (LD) refers to the extent of correlation between any two genetic variants in a population; complete LD (indicated by black blocks) indicates that the two variants are strongly correlated, i.e. they always occur together, and no LD (indicated by white blocks) indicates that the two variants do not occur together in the population more often than expected by chance.

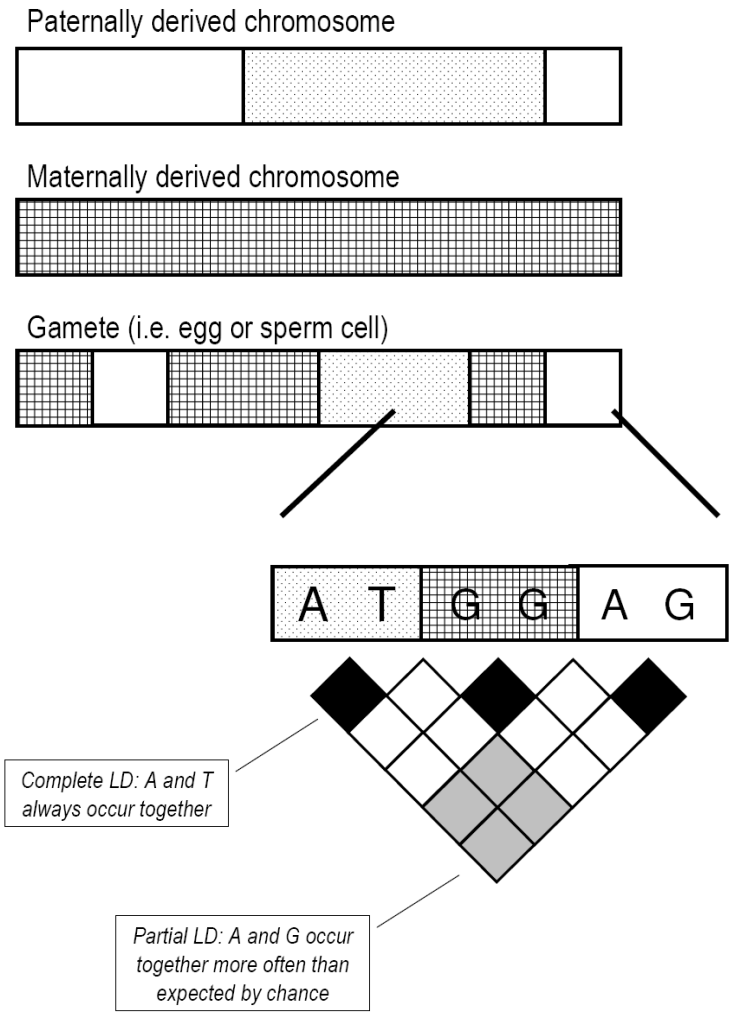

Figure 5: The principle of linkage disequilibrium - generation 2
This figure illustrates that over the course of many generations the levels of LD between any two genetic variants in the genome can vary (with darker blocks indicating higher LD). Recombination does not occur completely at random, there are several 'recombination hotspots' (Coop and Przeworski, 2007). The International HapMap project has mapped common patterns of LD in different ethnic populations (HapMap, 2003).

Genome-wide association (GWA) studies have cleverly made use of the concept of linkage disequilibrium (LD); per 'block' of DNA only one genetic variant needs to be genotyped to be able to infer (i.e. impute) the rest of the block based on pairwise LD. This principle is called 'tagging'; where key genetic variants are genotyped to efficiently capture the majority of common genetic variation within a region. It has been estimated that approximately 500,000 tags are needed to

cover all genetic variants in the human genome (Cichon et al., 2009); hence the genotyping of these tags is referred to as genome-wide genotyping. This immediately also clarifies the limitations of genome-wide studies, a) the 'tag' is strongly correlated with the ungenotyped genetic variations but not fully, thus any true disease associations would be diluted (or underestimated) if the pairwise correlation between the genotyped tag and the ungenotyped disease variant is e.g. 0.8 instead of 1.0, b) rare variants, only occurring in some individuals or some subgroups of the population, can not be inferred using this method, c) when testing 500,000 genetic variants for association, many variants will be associated with the disease or trait of interest by chance and it has proven to be very challenging to filter the true positives from the false positives, i.e. 'to separate the wheat from the chaff'.

Because 'genetic variants' and 'genome-wide assocation' are quite abstract concepts I wanted to attempt an analogy to clarify them. Let's say we are interested in the difference between commuters from Blackfriars train station in London (our cases) and commuters from London Bridge train station (our controls). We decide to study 1,000 individuals and we record 500,000 colours (our SNPs) per individual, namely eye colour, skin colour, hair colour, coat colour, wallet colour, purse colour, shoe colour, sock colour, etcetera, etcetera, etcetare. It will not be surprising that – by chance – some of the colours will appear at different frequencies between the commuters at Blackfriars and the commuters at London Bridge, e.g. London Bridge commuters may wear red socks significantly more often than Blackfriars commuters. The example of coloured socks may not be as ridiculous as it may sound; it is not unlikely that the average commuter at Blackfrairs, e.g. a banker working in the City of London, wears black socks more often than the average commuter at London Bridge, perhaps a nurse from the nearby Guy's hospital who tends to wear white socks. The colour of the socks may thus be a good 'tag' for the profession of the commuter (let's say our 'disorder' of interest). The quality of the tag is however important; success

depends on how well the tag tags the cause. In the example, sock colour may significantly be associated with the profession of the commuter (bankers wear black socks more often than nurses), but the effect size of sock colour in relation to profession, or the predictive value of sock colour when screening commuters, may be poor.

For the study of genetics of eating disorders it is important to note that a) a single nucleotide polymorphism (SNP), studied in genome-wide association (GWA) studies, is merely a 'tag' of common genetic variation in LD with the tagging SNP; i.e. the tagging SNP is not necessarily the causal genetic variant, and b) human assesement of the biological plausibility of the association is vital to 'separate the wheat from the chaff'.

1.3.4.2. Where it all began

In 2005 Klein and colleagues published the first genome-wide association (GWA) study (Klein et al., 2005); they tested genome-wide genetic variations in 96 cases and 50 controls for asscociation with age-related macular degeneration, a major cause of blindness in the elderly. They genotyped 103,611 SNPs of which the association of two SNPs passed a conservative Bonferroni correction for multiple testing (Klein et al., 2005). One of the two associated SNPs was missing in 21 out of 150 individuals, and its distribution violated the Hardy Weinberg Equilibrium (the latter will be explained in detail in the next chapter, paragraph 2.5.3 *Reference population, and Hardy Weinberg Equilibrium*, page 82), which clearly indicated that this was a spurious result caused by technical genotyping errors (Klein et al., 2005). Klein *et al* went on to resequence the genomic region of the other associated SNP, which included the gene 'complement factor H (CFH)' (a key regulator of the complement system of innate immunity), and found a total of 50 genetic variations in the region, of which three were non-synonymous (i.e. variations potentially affecting the protein functionality). Of these three non-synonymous SNPs a tyrosine-histidine variation was found to be

most strongly associated with the phenotype, and considered the 'causal variant', i.e. the variant causing the initial association, and the variant posing a risk factor in the development of age-related macular degeneration (Klein et al., 2005). Klein *et al* proved biological plausibility of this association by showing that various components of the complement cascade were present in affected eyes of patients, leading to the hypothesis that age-related macular degeneration results from an aberrant inflammatory process that includes inappropriate complement activation (Klein et al., 2005).

This spectacularly successful approach was rapidly copied by other researchers in other fields of science. Since 2005 more than 700 GWA studies have been published, more than 1500 genetic variations have successfully been associated with 101 human diseases and 124 traits (including height, and body weight or body mass index) (Kim et al., 2011), and the technique, data management, and statistical analyses of these fast amounts of data have been improved continously. Unfortunately not all studies were as successful as the one by Klein *et al* (Klein et al., 2005), hence in 2009 the Psychiatric GWAS Consortium Coordinating Committee published guidelines on GWA studies in psychiatry specifically (Cichon et al., 2009). The authors concluded a) that most genetic variation between any two individuals consisted of common (present in more than 5% of the population) single nucleotide polymorphisms (SNPs), and b) that most new findings were variants with a small increase in risk (relative risk between 1.12 and 1.2), and c) given the risk of false positive results, as a consequence of multiple testing, a genome-wide significance threshold of approximately 5×10^{-8} (5.00E-08) should be honoured (Cichon et al., 2009). Hence GWA studies would have to be performed using large sample sizes, of 8,000 to 12,000 cases and controls, or more (Cichon et al., 2009). This daunting number of cases required for analyses has not held back researchers; large-scale world-wide collaborations have been initiated and the first successful results have been published, or will be published shortly (State and Levitt, 2011,Kim et

al., 2011) (note: the Genetic Consortium for Anorexia Nervosa (GCAN) will publish their first results in 2012).

The proof of principle of the genome-wide technique comes from the results of the genetic studies of human height. It will be no surprise, even without calculations of heritability estimates, that height is a heritable trait (McEvoy and Visscher, 2009). Which genes accounted for this heritability was however not exactly known. GWA studies have now identified more than 50 genes or regions of the DNA associated with height (McEvoy and Visscher, 2009), and many of these genes are known to be involved in the development of bone and cartilage; they thus have obvious links to the regulation of observed height. Also, rare mutations in some of these genes had been linked to severe monogenic stature disorders, again supporting a role for the associated genes in normal height regulation (McEvoy and Visscher, 2009). Interestingly however, only 5 to 10% of the heritability of height has been explained so far (McEvoy and Visscher, 2009,Visscher et al., 2011). With height having a less debated and more obvious heritability than most psychiatric disorders, this is encouraging for genetic studies of psychiatric disorders; i.e. it illustrates the difficulty of identifying genetic variants underlying any trait, and should encourage researchers in the field of psychiatry not to give up on such a promising method, despite the daunting sample sizes and the negative results (Visscher et al., 2011).

1.3.4.3. Undetected heritability and genetic architecture

The heritability estimates of disorders and traits have been the main driving force behind genetic studies, i.e. the increased prevalence of disorders among first degree relatives of probands, and the general resemblance between relatives, have prompted researchers to hunt for the genes that may underly disorders or traits (also see paragraph 1.3.1 *Heritability*, page 37). The fact that only a small percentage of the heritability estimates have been explained, after analysing the DNA of thousands of cases (and spending much time and money),

has been used to critise genome-wide studies, and genetic studies in general. The success of these studies (also see pararagraph 1.3.4.2 *Where it all began*, page 50) is apparently overlooked by these critics. Indeed much of the heritability is unexplained as yet, but it does not imply that genetic studies have failed. Much has been learned about the genetic architecture of complex disorders and traits through these 'failed' studies, and many important new insights have been gained (Manolio et al., 2009,State and Levitt, 2011,Visscher et al., 2011,Day and Loos, 2011). For the genetic studies of EDs this actually presents an opportunity; based on the results of other psychiatric disorders, and other complex traits (such as body mass index), appropriate technologies and sample sizes can be chosen to optimise the chances of high-confidence results.

There are many possible reasons for the missing or, probably more appropriate, undetected (Yang et al., 2011,Lango et al., 2010,Davies et al., 2011,Visscher et al., 2011) heritability. These reasons include imprecise phenotyping, inadequate accounting for shared environment in twin estimates of heritability, common genetic risk factors with (very) small effect sizes, rare genetic risk factors, gene-gene interactions, gene-environment interactions, epigenetics, and an underestimated effect size due to causal variants being in less than full LD with the tagging variant (Manolio et al., 2009,Hebebrand et al., 2010,Kim et al., 2011,Davies et al., 2011,Visscher et al., 2011,Day and Loos, 2011). All of these factors are probably involved, but the rare versus common genetic risk factors have been one of the most debated topics (Uher, 2009,Visscher et al., 2011). Uher argues that mental illnesses associated with (strong) reproductive disadvantage are likely to have a large contribution from rare variants of recent origin (Uher, 2009), and indeed in autism (associated with strong decreased reproductive fitness (State MW and Levitt, 2011)) there are a substantial number of well reported cases resulting from *de novo* (new, i.e. recent) mutations, and more than 300 rare autism spectrum related genetic variations have now been identified (State and Levitt, 2011). However, as State and Levitt also point out, it

is incredibly challenging to identify genes for behavioural, cognitive, and emotional phenotypes; neurodevelopmental processes are regulated by thousands of genes, with different functions at different timepoints in the development and functioning of the human brain (Rakic, 2009,State and Levitt, 2011). For example, the genes found to be associated with autism tend to converge on alterations in the assembly and functioning of synapses (i.e. neuronal connections), and given the general non-specific function of these genes it is not surprising that the mutations associated with autism have also been found in individuals with more general non-specific social disabilities, language delay, selective mutism, and anxiety (reviewed by State and Levitt (State and Levitt, 2011)). Moreover, because of the importance of key processes such as synaptic functioning (i.e. the core process of brain functioning), there is much redundance in the biological processes involved, notably supported by the fact that these rare autism-related mutations also occur in non-affected individuals, including non-affected relatives (State and Levitt, 2011).

The negative selective pressure on the genetic risk factors underlying disorders with strong reproductive disadvantage as Uher describes it (Uher, 2009) may however not correlate with the forces that have shaped human genetic variation throughout human evolution (Manolio et al., 2009,Visscher et al., 2011). Manolio *et al* (Manolio et al., 2009) describe for example that type 1 diabetes, also associated with poor reproductive fitness prior to the discovery of insulin therapy, has been successfully associated with common genetic risk variants when large sample sizes were analysed (Barrett et al., 2009). Visscher *et al* (Visscher et al., 2011) agree with Uher that most causal variants are expected to be rare, and that mutations with a large effect on the incidence of disorders such as schizophrenia and anorexia nervosa (AN) have a negative effect on fitness, and that selection will thus eliminate these variants, or at least keep them at a low frequency in the population (Visscher et al., 2011). However Visscher *et al* also explain that mutations can have an effect on many phenotypes, and the

direction of the effect is not always the same for all traits, and has not always been the same throughout human evolution (Visscher et al., 2011), arguably the best example of this is the genetic mutation that increases the risk for sickle cell anaemia, but decreases the risk for malaria (Williams and Obaro, 2011). Despite the debate all researchers appear to agree that both common and rare variants are expected to be important in the aetiology of disease, and that unequivocal empirical evidence – yet to be obtained – will demonstrate to which extent either of the genetic risk variants contribute to the aetiology of complex disorders including EDs (Uher, 2009,Manolio et al., 2009,State and Levitt, 2011,Kim et al., 2011,Visscher et al., 2011). It is however clear that, given the results in the field of body mass index (Day and Loos, 2011), human height (Lango et al., 2010), human intelligence (Davies et al., 2011), and psychiatry in general (Cichon et al., 2009), that a polygenic genetic architecture consisting of many genetic risk factors each with a very small effect size (odds ratios in the range of 1.1 to 1.2) is a likely possibility for the genetic architecture of EDs.

1.4. Thesis aim and outline

It is clear that genetic risk factors are relevant in the aetiology of eating disorders (EDs). There is an increased prevalence of EDs in first degree relatives of sufferers, and twin studies estimate that approximately 50% of the phenotypic variance is accounted for by genetic factors. Which genetic risk factors cause the liability to EDs has however not yet been established. Therefore, the aim of this thesis was to identify the genes underlying the liability to EDs.

Over the past years several genetic studies of EDs have been conducted. Most of them have focussed on candidate genes and on anorexia nervosa (AN). Unfortunately the results were typically non-significant or non-replicated. For this reason more recent studies have tried to increase sample sizes and look at genes across the genome. The results of these larger-scale genetic studies have

not yet been confirmed; hence the aim of Chapter 1 of this thesis was to replicate the results of the most extensive genetic studies of AN done thus far.

Because of the complexity of the clinical presentation of EDs – and of psychiatric disorders in general – it has been suggested to focus on endophenotypes rather than clinical diagnoses. An endophenotype is a intermediate phenotype; it bridges between the complex clinical presentation and the underlying aetiology by 'breaking' the disorder up into manageable 'pieces' which can be studied on their own. Identifying genetic risk factors for endophenotypes has been hypothesized to be more straightforward and more successful (Gottesman and Gould, 2003). Drive for thinness (DT), bulimia (B), and body dissatisfaction (BD) as measured by the Eating Disorder Inventory (EDI) (Garner, 1983) are candidate endophenotypes for EDs. Therefore, Chapter 3 and Chapter 4 of this thesis focus on the range of DT, B, and BD scores in females from a general population sample. When studying endophenotypes, or traits, it is important to take note of the context in which they were measured. In Chapter 3 therefore the correlates of the DT, B, and BD scores in this population were discussed, and specifically the relation between the scores and body mass index was addressed.

Genetic risk variants for a disorder have an effect on biological processes by affecting normal gene functioning. Many different genetic variants could theoretically affect the same biological process, for example mutations affecting a receptor-ligand, mutations affecting a receptor, and mutations affecting an effector, could all attenuate a process of signal transduction. Hence, from a biological point of view it is plausible that different genetic risk variants cumulatively cause a disorder. The effects of individual genetic risk variants could even be too small to pick up in reasonable sample sizes without aggregating them into genes and pathways and testing their combined effect (Cantor et al., 2010). For this reason Chapter 4 focussed on secondary genome-wide genetic

analyses: individual genetic variants were combined per gene and per pathway, and their combined effect was tested for association with the DT, B, and BD scores.

The overall conclusion and discussion of the results from the different studies in this thesis was presented in Chapter 5, including an outline of future directions for the genetic studies of eating disorders.

Molecular genetic analysis of Anorexia Nervosa

2. Molecular genetic analysis of Anorexia Nervosa

2.1. Literature background

Genetic studies on Anorexia Nervosa (AN) have mainly focussed on candidate genes up until now. The results of these studies have not been consistently replicated although some associations remain interesting (also see paragraph 1.3.3 *Candidate gene studies*, page 42). As recommended by the Psychiatric GWAS Consortium Coordinating Committee (Cichon et al., 2009) individual research groups are now combining their efforts in order to obtain large enough sample sizes to gain the statistical power needed to identify genetic risk factors for AN. This is because most new findings in other psychiatric disorders or complex traits have been variants associated with a small increase in risk (relative risk 1.12 to 1.2) (Cichon et al., 2009,Yang et al., 2011,Kim et al., 2011,Davies et al., 2011,Visscher et al., 2011).

Researchers in the field of eating disorders (EDs) are also adopting large-scale genetic association methods. Examples of multisite collaborative study groups organized for the purpose of genetic studies in EDs include: the Japanese Genetic Research Group for Eating Disorders (JGRED), the Price Foundation Genetic Studies of Eating Disorders, and the Genetic Consortium for Anorexia Nervosa (GCAN). The results of their collaborations have been published recently and include both case-control study designs (Nakabayashi et al., 2009,Pinheiro et al., 2010,Wang et al., 2011) as well as within-case study designs (Root et al., 2011). The genome-wide study by the GCAN consortium is currently in the final phase of analyses and is expected to be published in 2012. Large-scale genetic studies can be complemented by meta-analyses of existing data. Brandys *et al* performed two such studies for AN (Brandys et al., 2011) (also see paragraph 1.3 Genetics of

eating disorders, page 37) and (Brandys et al., Anorexia nervosa and the Val158Met polymorphism of the COMT gene: meta-analysis and new data, in press).

This chapter of the thesis was designed to replicate the results of some of the most extensive collaborative studies done on AN thus far; namely, the most significantly associated haplotypes from a large-scale candidate gene study of AN (Pinheiro et al., 2010); one of the top hits from the first genome-wide association (GWA) study by Wang *et a* (Wang et al., 2011)); and the most recent meta-analysis by Brandys *et al* (Brandys et al, in press).

2.1.1. Replication of the most extensive genetic studies of AN

Pinheiro *et al* conducted the most extensive hypothesis-driven candidate gene study of AN done thus far (Pinheiro et al., 2010); they studied 5,151 SNPs in 182 genes, selected on the basis of previous association, gene expression in the brain, biological plausibility, and markers from regions linked to AN in family based study designs (also see 1.3 Genetics of eating disorders, page 37). After accounting for multiple testing there were however no significant results (Pinheiro et al., 2010). In order to increase their sample size and statistical power, our group was asked to replicate some of their top findings, specifically their top haplotypes (personal communication). Pinheiro *et al* defined haplotype blocks for the 5,151 SNPs in their study by using the TAGGER method in Haploview 4.0 (Pinheiro et al., 2010). This algorithm creates haplotype blocks when 95% of informative comparisons are in strong linkage disequilibrium (LD), i.e. it creates blocks of SNPs that are likely to have been inherited together (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45). The blocks tested for association with AN ranged in size from two SNPs to 79 SNPs, none of the haplotypes were significant following correction for multiple testing, but the top 25 haplotypes were published as Supplementary material (Pinheiro et al., 2010)). The haplotypes chosen for replication were three-SNP

haplotypes from two genes, namely phenylalanine-4-hydroxylase (PAH) and solute carrier family 18 A1 (SLC18A1) (see Table 2).

A three-SNP haplotype (i.e. a haplotype consisting of three SNPs) could theoretically occur in eight combinations (see Table 3 for an example), in which case it would be analysed as having seven degrees of freedom (DF). Not all combinations are equally common though, and in haplotype analyses a zero-frequency threshold can be set, i.e. very rare haplotype combinations can be considered 'non-occuring', in which case the DF will be less than seven (e.g. the top haplotype by Pinheiro *et al* was analysed with three DF i.e. only four haplotype combinations were considered, see Table 2). It is important to note that the 'phase' of the haplotypes is estimated; each chromosome has two strands of DNA (a paternal and a maternal strand), and a haplotype is a block of DNA that is inherited on a single strand of DNA (Browning and Browning, 2011). For technical reasons, from raw genotyping data, it can not be determined from which DNA strand any allele originates, hence haplotype combinations are inferred; their frequency is estimated (Browning and Browning, 2011).

Pinheiro *et al* - published

Rank of haplotype	*Chromosome*	*Position*	*CHISQ*	*DF*	*P*	*SNP1*	*SNP2*	*SNP3*
1	12	101735233	20.89	3	0.0001111	rs1801153	rs1718312	rs12831013
9	8	20087205	13.34	3	0.003949	rs2173114	rs7820517	rs987778
17	8	20086029	14.29	4	0.006434	rs7836907	rs2173114	rs7820517

Table 2: Results of Pinheiro *et al* for replication (adapted from (Pinheiro et al., 2010))
Three haplotypes out of the top 25 published haplotypes (Pinheiro et al., 2010) were chosen for replication. Haplotype #1 is located in the gene PAH, haplotypes #9 and #17 are located in SLC18A1 (also see Table 6, page 65). Results were communicated to us personally prior to publication, but some changes were made to the manuscript resulting in slightly different results in the published paper. By this time my laboratory work had commenced, hence only three published haplotypes (#1, 9, and 17) could be replicated. Degrees of freedom (DF) indicates that not all possible haplotype allele combinations were observed; four haplotype combinations were tested for haplotype #1 and #9 (DF = 3), and five combinations for haplotype #17 (DF = 4).

Theoretical combinations for a three SNP (A/T) haplotype

	SNP#1	*SNP#2*	*SNP#3*
1	A	A	A
2	A	T	A
3	A	T	T
4	A	A	T
5	T	A	A
6	T	A	T
7	T	T	T
8	T	T	A

Table 3: Example theoretical combinations of a three-SNP haplotype
A SNP has two alleles (e.g. A or T), thus a three-SNP haplotype can theoretically occur in eight different combinations. Generally not all combinations are equally common, and thus in haplotype association analyses only the number of observed combinations is taken into account, with sometimes a set 'zero-frequency threshold' for very rare haplotypes.

The top result from the first genome-wide association (GWA) study of AN was communicated to us personally prior to publication (personal communication). However the authors added more cases to their sample prior to final publication, resulting in slightly different results in their published paper (Wang et al., 2011); by this time my laboratory work had already commenced, hence only the number four SNP (rs2383378, A-kinase anchor protein 6 (AKAP6) gene, see Table 4) of the published paper (Wang et al., 2011) was included in this chapter. None of the SNPs tested by Wang *et al* reached genome-wide significance (Wang et al., 2011); the p value in Table 4 is the p value uncorrected for multiple testing (Wang *et al* tested ~600,000 markers for association with AN (Wang et al., 2011), a Bonferroni threshold for multiple testing, with α set at 0.05, would be 0.05 / 600,000 = 8.3E-08).

Wang *et al* - published

Rank of SNP	*SNP*	*Locus*	*Gene*	*MAF case*	*MAF control*	*P-value*
4	rs2383378	14q12	AKAP6	0.35	0.41	6.41E-06

Table 4: Results of Wang *et al* for replication (adapted from (Wang et al., 2011))
This SNP (rs2383378) was initially the top result with a p value of 3.8E-07 (personal communication), however prior to publication more samples were added and the results changed slightly; in the published paper this SNP is the number four SNP overall (Wang et al., 2011). MAF refers to Minor Allele Frequency, i.e. the allele of the SNP which occurs least frequently in the population.

Brandys *et al* published a negative result, their data and meta-analysis indicate that the SNP rs4680 of the catechol-O-methyltransferase (COMT) gene is not associated with AN (Brandys et al, in press) (see Table 5). The two different alleles of this SNP (adenine (A) and guanine (G)) lead to a different amino-acid in the protein-product of the gene at position 158, also known as a "misssense" or "nonsynonymous" polymorphism. The A allele of the SNP is the Methionine (Met) allele, and the G allele is the Valine (Val) allele, hence the SNP is also known as the Val158Met polymorphism of the COMT gene. The Met allele causes the COMT protein to be less stable, resulting in lower protein activity, and this particular polymorphism has been associated with several psychological disorders including substance abuse, schizophrenia, obsessive-compulsive disorder (OCD), and bipolar disorder (see (Hosak, 2007) for a general review, and (Rask-Andersen et al., 2010a) for an ED specific review). Brandys *et al* analysed data from a Dutch (Leiden and Utrecht) cohort and performed a meta-analysis on 2,021 cases and 2,848 controls (Brandys et al, in press). The results of the Utrecht cohort were suggestive of a dominant genotypic effect of the Met-allele (genetic dominance indicates that one or two copies of the allele increase the risk equally, i.e. only one copy is sufficient to increase the risk), but the meta-analysis indicated that overall there was no association (meta-analysis dominant genotypic effect p= 0.18, see Table 5, Brandys et al, in press).

Brandys *et al* - in press

	SNP	*Gene*
	rs4680 (Val158Met)	COMT
Results - allelic contrast	*OR*	*p*
Utrecht cohort	1.14	0.14
Leiden cohort	1.02	0.85
Meta-analysis	1.03	0.42
Results - dominant effect Met allele		
Utrecht cohort	**1.42**	**0.03**
Leiden cohort	1.00	1.00
Meta-analysis	1.10	0.18

Table 5: Results of Brandys *et al* for replication (Brandys et al, in press)

The meta-analysis by Brandys *et al* provides evidence that the Val158Met variant of the

COMT gene is not associated with AN. The only indication of association of this variant was under the dominant model of genetic effect in the Utrecht cohort (OR 1.42, p=0.03, in **bold**), however this effect was non-significant in the Leiden cohort, nor was it significant in the meta-analysis (Brandys et al, Anorexia nervosa and the Val158Met polymorphism of the COMT gene: meta-analysis and new data, in press).

2.2. Aim and outline

The SNPs and haplotypes of interest were tested for association with AN using both a case-control and a within-case study design. The case-control analyses included a sample from London (United Kingdom), and sample from Vienna (Austria). The within-case analyses included samples from London, Vienna, and Utrecht (The Netherlands). The within-case study design tested association with lowest adult lifetime body mass index (BMI) following the criteria outlined by Bulik *et al* in 2005 (Bulik et al., 2005) and Root *et al* in 2011 (Root et al., 2011), and additionally within-case analyses on highest adult lifetime BMI were conducted because the results of Chapter 3 of this thesis indicate that highest adult lifetime BM is a predictor of disordered eating (see paragraph 3.5 *Results*, page 127). Haplotype SNPs were present as single SNPs as well as haplotype results, though it should be noted that they were only published as haplotypes associated with AN, not as single SNPs. My laboratory work was commenced following personal communication of the results, however prior to publication the authors made some amendments to their work and for this reason only nine out of initially 26 selected SNPs were published (see Table 6) (Pinheiro et al., 2010,Wang et al., 2011,Brandys et al., in press). The methods section will include all 26 SNPs since the genotyping method analyses 26 SNPs in parallel, however in the results section only the nine published SNPs will be presented; an overview of all SNP results will be presented in the Appendix (see page 232).

Pinheiro et al				
SNP rs#	*Chromosome*	*Position*	*Allele*	*Description of gene*
rs1801153	12	101756896	C/T	Phenylalanine-4-hydroxylase (EC 1.14.16.1) (PAH) (Phe-4- monooxygenase)
rs1718312	12	101765318	A/G	Phenylalanine-4-hydroxylase (EC 1.14.16.1) (PAH) (Phe-4- monooxygenase)
rs12831013	12	101769366	C/G	Phenylalanine-4-hydroxylase (EC 1.14.16.1) (PAH) (Phe-4- monooxygenase)
rs7836907	8	20041749	A/T	SLC18A1 solute carrier family 18 (vesicular monoamine)
rs2173114	8	20042925	C/G	SLC18A1 solute carrier family 18 (vesicular monoamine)
rs7820517	8	20044636	A/T	SLC18A1 solute carrier family 18 (vesicular monoamine)
rs987778	8	20046580	A/C	SLC18A1 solute carrier family 18 (vesicular monoamine)
Wang et al				
rs2383378	14	32352221	A/C	A-kinase anchor protein 6 (Protein kinase A-anchoring protein 6) (PRKA6)
Brandys et al				
rs4680	22	18331271	A/G	Catechol O-methyltransferase (EC 2.1.1.6)

Table 6: Overview of SNPs for replication
Three haplotypes published by Pinheiro *et al* (Pinheiro et al., 2010) will be tested for association with AN; one haplotype from chromosome 12, and two (partly overlapping) haplotypes from chromosome 8.

2.3. Methods

2.3.1. Sample collection

The AN cases examined in this study were recruited from three different locations: London (United Kingdom (UK)), Utrecht (The Netherlands) and Vienna (Austria). Approval from the local ethical committees was in place and informed consent was obtained from all study participants. Cases were included in the study if they fulfilled the following criteria (which match the criteria of the Genetic Consortium for Anorexia Nervosa (GCAN)):

- *Inclusion:* Cases with a lifetime diagnosis of AN (restricting or binge/purge subtype) excluding amenorrhoea (based on DSM-IV, assessed by structured clinical interviews). The DSM-IV amenorrhea criterion was omitted because the use of oral contraceptives and uncertain menstrual history make this criterion difficult to assess, moreover it has been shown that it does not increase the diagnostic specificity (Gendall et al., 2006,Poyastro et al., 2007).
- *Exclusion:* Any medical or psychiatric condition that may confound the diagnosis of AN including psychotic disorders, severe learning disabillities,

and other medical and neurological conditions causing weight or appetite loss.

UK controls were recruited as controls for the Bipolar Association Case-Control Study (BACCS) and screened for absence of lifetime psychiatric disorders (mean age 32 years, 40:60 male:female ratio). Austrian controls were recruited as controls for a childhood migraine study (mean age 10 years, 50:50 male:female ratio, (Wober-Bingol et al., 2011)); they were screened for migraine but not for eating disorders. A concern on using controls not screened specifically for eating disorders is the potential loss of power due to inclusion of controls with latent eating disorder diagnoses, however this loss of power is minimal given the low lifetime prevalence of AN (0.6%, (Jacobi et al., 2004b,Hudson et al., 2007,Swanson et al., 2011)). No controls were available for the Utrecht sample; the Utrecht sample was thus only part of the within-case analyses. All cases and controls were of Caucasian (central and western European) ancestry.

2.3.2. DNA extraction

DNA was extracted from blood and from cheek swab samples using an in-house variation of the phenol-chloroform extraction (cases and controls from London and Vienna (Freeman et al., 1997,Freeman et al., 2003)), Nucleon BACC II kits by GE Life Sciences (London cases), a salting-out procedure (Utrecht cases, (Miller et al., 1988)), and Genecatcher kits by Invitrogen (Utrecht cases).

2.3.3. Genotyping

Genotyping was done using the Sequenom iPlex Gold reaction. The iPlex Gold reaction is a universal method for detecting insertions, deletions, substitutions, and other polymorphisms in DNA. The first step of the iPlex Gold reaction is a polymerase chain reaction (PCR) which amplifies the DNA region of interest. Next, any unincorporated nucleotides (i.e. DNA building blocks; deoxyribonucleotide triphosphates (dNTPs); A, T, C and G) are neutralised using shrimp alkaline phosphatise (SAP). The SAP cleaves off a phosphate molecule

converting the dNTPs to dNDPs, this inactivates them and prohibits further reaction. Next, iPlex Gold reaction cocktail (primer, enzyme, buffer, and mass-modified nucleotides) is added to the amplified DNA. This reaction will incorporate a mass-modified nucleotide into the exact site of interest (i.e. the location of the SNP); the two different alleles of the SNP will then be distinguishable by their mass. The amplified DNA and iPlex Gold reaction cocktail are thermocycled (discrete, pre-programmed steps in which the temperature of the reaction is raised and lowered alternately) to process the reaction. In the reaction mixture, all four nucleotides -A, T, C, and G- (mass-modified) are present.

In the final step the products of the iPlex Gold reaction are desalted and transferred onto a SpectroCHIP by the MassARRAY nanodispenser machine. The SpectroCHIP is then analyzed by the MassARRAY mass spectrometer using time-of-flight (TOF) analysis; the DNA particle will 'fly' through the mass spectrometer with higher mass DNA 'flying' more slowly than lower mass DNA. Each allele of each SNP is designed to have a different mass; they will thus all 'fly' through the mass spectrometer at a different speed and pass the detector of the mass spectrometer at a different time. Each time a DNA particle passes the detector a peak will appear on the mass spectrum, with higher peaks indicating more DNA. Since the mass of each SNP is known the location of its corresponding peak on the mass spectrum is known too. Per SNP three distinct peaks can arise; one for each allele and one for the primer peak (the latter will only be present in case the iPlex Gold reaction failed). Figure 6 shows the details of the iPlex Gold reaction.

[image unavailable in e-thesis]

Figure 6: Sequenom iPlex Gold reaction
Following DNA amplification any in the amplified DNA (PCR product) left-over unincorporated dNTPs (i.e. DNA building blocks: A, T, C and G) were inactivated by SAP treatment. Next, the iPlex Gold reaction incorporates a mass-modified nucleotide into the exact site of interest (i.e. SNP site), rendering allele-specific DNA products with distinguishable masses. After mass spectrometry time-of-flight analyses (MALDI-TOF) three distinct peaks can arise per SNP, one for each allele and one for the primer peak (the latter will only be present in case the iPlex Gold reaction failed) (the image was obtained from (Gabriel et al., 2009)).

The Sequenom iPlex gold reaction can analyse 26 SNPs in parallel. The design of the Sequenom panel must be such that each allele and each primer peak have a distinct mass, and that primers do not overlap in sequence as this would cause

unspecific binding. Figure 7 and Figure 8 show the design of the current study; there are three possible peaks (i.e. masses) per SNP, and 26 x 3 = 78 peaks in total.

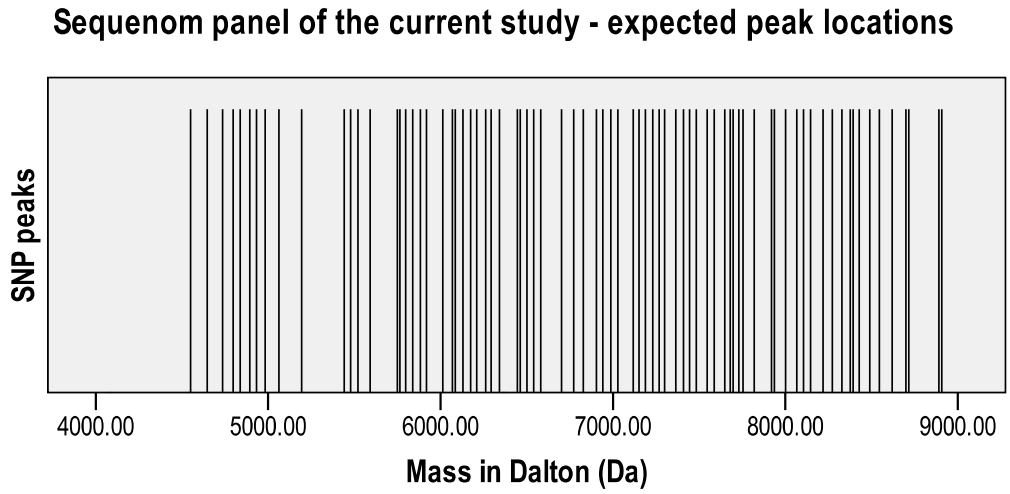

Figure 7: Sequenom panel design of the current study
Each peak represents allele 1, allele 2, or the primer peak of a SNP (number of SNPs is 26, total number of peaks is 26 x 3 = 78). The mass is expressed in units of Dalton (Da).

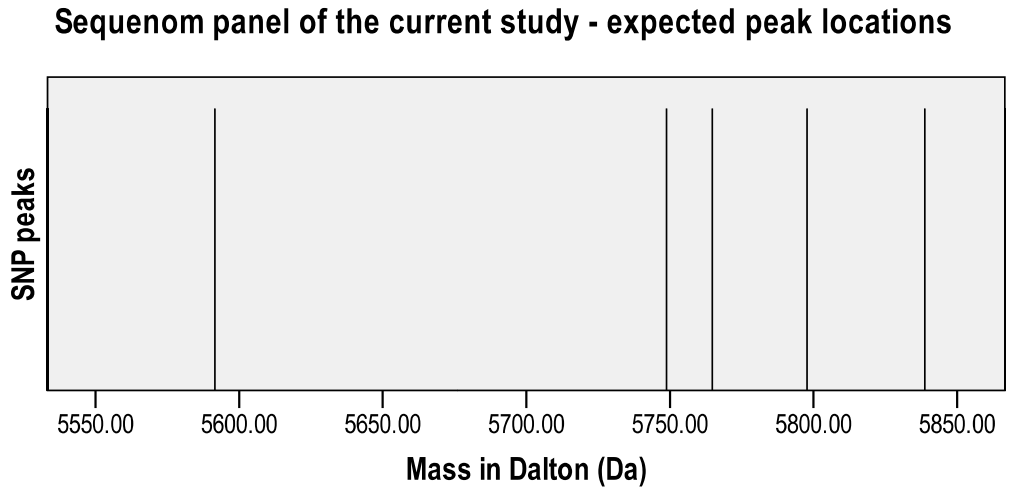

Figure 8: Sequenom panel - zoom of the 5700 Da region
This zoom-in of the Sequenom panel shows five out of 78 peaks: rs1390939 (primer, 5591 Da), rs4633 (allele 1, 5748 Da), rs4633 (allele 2, 5764 Da), rs12728678 (primer, 5797 Da) and rs1390939 (allele 1, 5838 Da).

2.4. *Sample quality control*

A total of 2,304 samples were run on Sequenom, they were run on six 384 well plates in four batches. The plates included within and between plate and batch duplicates and negative controls (NTCs) (see Table 7).

Type of sample	*n individuals*	*n samples*
Unique samples	1,066	1,066
NTCs	-	119
Other technical controls	-	209
Duplicates	221	442
Triplicates	119	357
Quadruplicates	22	88
Quintuplicates	1	5
Sextuplicates	3	18
Total	*1,432*	*2,304*

Table 7: Overview of samples run on Sequenom
In total 2,304 samples were run on Sequenom including negative controls (NTCs) and duplicate samples for quality control purposes, these were samples of in total 1,432 unique individuals (cases and controls from London, Utrecht and Vienna).

2.4.1. Negative controls

In total 119 negative controls (NTCs) were included across all plates. Negative controls should be negative i.e. not have genotype calls, it is a quality control measure used to assess the quality of the laboratory work. Genotype calls in the NTCs would be an indication of DNA contamination; if this were the case genotyping results would be unreliable. NTCs consisted of double distilled water (ddH_20) instead of DNA and were treated exactly the same as the DNA samples. The error percentage was calculated by dividing the number of genotype calls by the total number of possible calls. From the error percentage per plate it becomes clear plate 6 is unreliable, as the error percentage is much higher than the other plates (36,92% vs a mean of 8%, see Table 8) and thus all samples from plate 6 have been excluded from analyses (n=58, see paragraph 2.4.3 *Overview of sample exclusions*, page 76). Other technical controls (n=209, see Table 7) were samples without DNA with only iPlex Gold reaction ingredients, e.g. primer,

enzyme, buffer, or mass-modified nucleotides. These were included on plate 6, and will not be considered further due to the high NTC error percentage on this plate. The mean error percentage across all samples, excluding plate 6, is 8% (ranging from 0 – 80%, most right column Table 8). Even though the error percentage is high there is no sign of DNA contamination; if there would be DNA contamination the high error rate would be consistent across SNPs (as happened on plate 6); all 26 SNPs were analysed at the same time and thus all SNPs would be affected equally by contamination. Looking in detail at the mass spectra of the NTCs it is apparent that the high error rate is caused by a marker specific design problem; the primer peak of some SNPs is located very closely to the allele peak location of another SNP, causing the primer peak to mistakenly be identified as an allele; this problem will be discussed in detail in paragraph 2.5 *SNP quality control*, page 77.

NTC error percentage

SNP rs#	plate 1	plate 2	plate 3	plate 4	plate 5	plate 6	All samples	All_excluding plate 6
4680	0.00%	0.00%	0.00%	0.00%	0.00%	40.00%	3%	0%
709611	0.00%	0.00%	0.00%	0.00%	0.00%	40.00%	3%	0%
1718312	0.00%	0.00%	0.00%	0.00%	0.00%	20.00%	2%	0%
2020933	0.00%	0.00%	0.00%	0.00%	0.00%	30.00%	3%	0%
2917928	0.00%	0.00%	0.00%	0.00%	0.00%	20.00%	2%	0%
11264262	0.00%	0.00%	0.00%	0.00%	0.00%	60.00%	5%	0%
17210001	0.00%	0.00%	0.00%	0.00%	0.00%	30.00%	3%	0%
363221	0.00%	0.00%	5.26%	0.00%	0.00%	50.00%	5%	1%
987778	0.00%	3.13%	0.00%	0.00%	0.00%	30.00%	3%	1%
1390939	0.00%	3.13%	0.00%	0.00%	0.00%	30.00%	3%	1%
1801153	0.00%	0.00%	5.26%	0.00%	0.00%	40.00%	4%	1%
2173114	0.00%	3.13%	0.00%	0.00%	0.00%	40.00%	4%	1%
3808955	0.00%	0.00%	5.26%	0.00%	0.00%	50.00%	5%	1%
7820517	0.00%	0.00%	5.26%	0.00%	0.00%	20.00%	3%	1%
7863731	0.00%	0.00%	5.26%	0.00%	0.00%	0.00%	1%	1%
10444117	0.00%	0.00%	0.00%	4.76%	0.00%	50.00%	5%	1%
11564771	0.00%	0.00%	5.26%	0.00%	0.00%	30.00%	3%	1%
12728678	0.00%	0.00%	5.26%	0.00%	0.00%	40.00%	4%	1%
1055663	0.00%	0.00%	0.00%	0.00%	9.52%	50.00%	6%	2%
12831013	0.00%	0.00%	0.00%	0.00%	9.52%	40.00%	5%	2%
7836907	0.00%	6.25%	0.00%	0.00%	9.52%	30.00%	6%	4%
1126758	0.00%	3.13%	10.53%	9.52%	4.76%	20.00%	7%	6%
363251	0.00%	0.00%	5.26%	9.52%	28.57%	40.00%	11%	8%
6355	0.00%	40.63%	0.00%	28.57%	57.14%	50.00%	30%	28%
2383378	100.00%	21.88%	10.53%	95.24%	90.48%	30.00%	56%	59%
4633	100.00%	68.75%	36.84%	100.00%	100.00%	80.00%	80%	80%
Mean	**7.69%**	**5.77%**	**3.85%**	**9.52%**	**11.90%**	**36.92%**	**10%**	**8%**
n	**16**	**32**	**19**	**21**	**21**	**10**	**119**	**109**

Table 8: Negative control (NTC) error percentage, per SNP, per plate
SNPs are sorted on error percentage, high error rates are shown in grey. Error rates were consistently high on plate 6 indicative of DNA contamination; results of plate 6 are

thus unreliable and were excluded from analyses. The mean error percentage across samples, excluding plate 6, is 8% (most right column, also see paragraph 2.5, page 77). N refers to the number of NTCs.

2.4.2. Duplicates

In total 221 duplicate pairs, 119 triplicates, 22 quadruplicates, one quintuplicate, and three sextuplicates were analysed (see Table 7). This included within and between plate duplicates, exact duplicates (i.e. DNA from the same individual, the same blood tube, the same plate well), and regular duplicates (i.e. DNA from the same individual but from a different blood tube; either because two blood tubes were taken at the same occasion, or because the individual donated blood for research on multiple occasions). Duplicate samples were included as a quality control measure; genotype calls should match exactly between duplicates across plates. The number of duplicates included in the current study was higher than average for pragmatic reasons, a) to ensure even distribution of case and control samples from the different countries across plates, and b) to not leave any genotyping slots unused (Sequenom plates were filled completely).

The duplicate error percentage was calculated by dividing the number of mismatching duplicate genotypes by the total number of possible mismatching duplicate genotypes (idem for triplicates, etc). The duplicate error percentage on plate 6 was calculated separately because of the high NTC error percentage on this plate. The duplicate error percentage of plate 6 is only slightly higher than the mean duplicate error percentage of the other plates (1.32% vs 0.77%, see Table 9). This could be explained by the fact that a possible contamination would affect NTCs but not necessarily DNA samples because the original DNA would be in excess, plate 6 was nevertheless excluded from analyses. The mean duplicate error percentage excluding plate 6 is 0.77%, which is within the 1% technical error percentage of the Sequenom technique. The SNP with the highest duplicate error is also the SNP with the highest NTC error (rs4633; 5.43% and 80% respectively) (this will be discussed in detail in paragraph 2.5 *SNP quality*

control, page 77). For the other SNPs there is no apparent relation between duplicate error rate and NTC error rate (see Table 9).

Duplicate error percentage				NTC error percentage
SNP rs#	**plate 6**	**All_excluding plate 6**	**All duplicates**	**All_excluding plate 6**
4633	4.48%	5.84%	5.43%	80%
4680	1.49%	2.60%	2.26%	0%
6355	0.00%	0.00%	0.00%	28%
363221	0.00%	0.00%	0.00%	1%
363251	1.49%	0.65%	0.90%	8%
709611	1.49%	0.00%	0.45%	0%
987778	0.00%	0.00%	0.00%	1%
1055663	1.49%	1.30%	1.36%	2%
1126758	0.00%	0.65%	0.45%	6%
1390939	4.48%	1.30%	2.26%	1%
1718312	1.49%	0.65%	0.90%	0%
1801153	2.99%	0.00%	0.90%	1%
2020933	1.49%	0.00%	0.45%	0%
2173114	0.00%	0.00%	0.00%	1%
2383378	2.99%	1.95%	2.26%	59%
2917928	1.49%	0.65%	0.90%	0%
3808955	0.00%	0.65%	0.45%	1%
7820517	2.99%	0.00%	0.90%	1%
7836907	1.49%	0.65%	0.90%	4%
7863731	1.49%	0.65%	0.90%	1%
10444117	0.00%	1.30%	0.90%	1%
11264262	1.49%	0.00%	0.45%	0%
11564771	0.00%	0.00%	0.00%	1%
12728678	1.49%	1.30%	1.36%	1%
12831013	0.00%	0.00%	0.00%	2%
17210001	0.00%	0.00%	0.00%	0%
mean	***1.32%***	***0.77%***	***0.94%***	***mean*** ***8%***
n pairs	***67***	***154***	***221***	***n*** ***109***

Table 9: Duplicate error percentage per SNP
Plate 6 was calculated separately because of the high NTC error percentage on this plate. Overall the duplicate error rates are normal, only rs4633 has a high duplicate error rate (5.84% excluding plate 6, upper row). Total number of possible mismatches was 26 SNPs x 221 pairs = 5,746. The duplicate error rate excluding plate 6 was 0.77%. The NTC error percentage excluding plate 6 (most right column) was included for easy comparison of error rates on SNPs.

In total there were 28 out of 221 duplicate pairs with mismatching genotypes (number of mismatches per pair ranged from 1 to 12; total number of possible mismatches was 26 SNPs x 221 pairs = 5,746). Most mismatching samples only mismatched on one out of 26 genotypes. Of the samples mismatching on multiple genotypes all but two mismatches could be explained by the fact that one of the samples had many missing genotypes, i.e. one of the samples apparently contained little DNA. A sample can contain little DNA either because its DNA concentration is very low (in case two different blood samples from the

same individual were included, of which one had a low DNA concentration) or because of standard limitations of the laboratory work equipment used (given the small volume of DNA used for analyses, one microliter, some samples will have a lower DNA concentration by chance e.g. due to the occasional small air bubble). Even though the duplicate error percentage is within the technical error percentage of the technique, mismatching samples were examined in detail in order to ensure a high quality result of analyses. Two samples stood out for having many mismatching genotypes while there was no apparent difference in DNA concentration; sample MR151:01 (12 mismatches, London case sample) and AN185 (9 mismatches, Utrecht case sample); these two samples were excluded from analyses. As a general rule for duplicates the sample with the least missing genotypes was chosen for analyses, assuming this sample had the best DNA quantity and/or quality.

The overall triplicate error percentage is comparable to the duplicate error percentage (1.14%, see Table 10). The SNP with the highest triplicate error percentage is also the SNP with the highest duplicate error percentage and NTC error percentage (rs4633; 12.61%, 5% and 80% respectively). For the other SNPs there is no apparent relation between error percentages.

Triplicate error percentage					NTC error	Duplicate error
SNP rs#	Plate 6	All_excluding plate 6	All triplicates		All_excluding plate 6	All duplicates
4633	0.00%	13.89%	12.61%		80%	5%
4680	0.00%	0.00%	0.00%		0%	2%
6355	0.00%	1.85%	1.68%		28%	0%
363221	0.00%	1.85%	1.68%		1%	0%
363251	0.00%	0.93%	0.84%		8%	1%
709611	0.00%	0.93%	0.84%		0%	0%
987778	0.00%	0.00%	0.00%		1%	0%
1055663	0.00%	0.00%	0.00%		2%	1%
1126758	9.09%	0.93%	1.68%		6%	0%
1390939	18.18%	0.00%	1.68%		1%	2%
1718312	9.09%	0.00%	0.84%		0%	1%
1801153	0.00%	1.85%	1.68%		1%	1%
2020933	0.00%	0.00%	0.00%		0%	0%
2173114	9.09%	0.00%	0.84%		1%	0%
2383378	0.00%	0.93%	0.84%		59%	2%
2917928	0.00%	0.00%	0.00%		0%	1%
3808955	0.00%	0.00%	0.00%		1%	0%
7820517	9.09%	0.00%	0.84%		1%	1%
7836907	18.18%	0.00%	1.68%		4%	1%
7863731	0.00%	0.00%	0.00%		1%	1%
10444117	0.00%	1.85%	1.68%		1%	1%
11264262	9.09%	1.85%	2.52%		0%	0%
11564771	9.09%	0.00%	0.84%		1%	0%
12728678	0.00%	1.85%	1.68%		1%	1%
12831013	0.00%	0.93%	0.84%		2%	0%
17210001	0.00%	0.00%	0.00%		0%	0%
mean	***3.50%***	***1.14%***	***1.36%***	*mean*	***8%***	***0.94%***
n triplicate pairs	***11***	***108***	***119***	*n*	***109***	***221***

Table 10: Triplicate error percentage per SNP
Plate 6 was calculated separately because of the high NTC error percentage on this plate. Overall the triplicate error percentage is normal (mean triplicate error rate excluding plate 6 is 1.14%), only rs4633 stands out for having a high error percentage (12,61%, upper row). The NTC error percentage, and the duplicate error percentages were included for easy comparison of error rates on SNPs.

In total 12 out of 119 triplicate pairs mismatched (excluding SNP rs4633); the number of mismatches per pair ranged from 1 to 6. All mismatches could be explained by the fact that one of the samples either had many missing genotypes (as discussed for duplicates) or was run on plate 6. One sample stands out for having mismatching genotypes and many missing genotypes on all three Sequenom runs (sample EDE355, London case sample), because genotype calls might not be reliable when many genotype calls are missing this sample has been excluded from further analyses. This was the only sample excluded based on triplicate mismatching. As for the duplicates; as a general rule for triplicates the sample with the least missing genotypes was chosen for analyses. The same holds true for the quadruplicate, quintuplicate and sextuplicate pairs that were

included (see Table 7); three quadruplicates and one quintuplicate were mismatching (excluding SNP rs4633) and all mismatches could be explained by the fact that one of the samples had many missing genotype calls - thus none have been excluded.

2.4.3. Overview of sample exclusions

Samples on plate 6 were excluded because of the high NTC error percentage on this plate (see 2.4.1 *Negative controls* and Table 8); 57 unique samples and 1 duplicate sample were run on plate 6, these were all cases from the Utrecht sample apart from one sample from Vienna. Note: plate 6 did also contain London and Vienna case and control samples, however of these samples a duplicate sample on a different Sequenom plate was available. The samples MR151:01 and AN185 were excluded for having unexplained duplicate mismatches, and triplicate sample EDE355 was excluded since it had mismatching genotype calls and many missing genotype calls on every run. There were 38 samples with missing genotype calls on more than half of the SNPs (more than 13 out of 26 SNPs), these samples were excluded from further analyses because DNA quantity or quality apparently was poorly. Nine samples were excluded because they did not originate from central or western Europe (this included the mismatching EDE355). And finally three samples were excluded because they were related; GAN3301 was excluded because her sister participated in the study, and EHE4962 and AN551 were excluded because they had participated in multiple studies and were thus known under different ID codes of which the one with the most missing genotype calls was excluded. Total number of excluded samples was 110 (7.7%), 1,322 samples were tested for association with AN (see Table 11 for details).

Overview of exclusions	*n start*	*n exclusions*	*n final*
unique samples	1066	84	982
duplicate pairs	221	16	205
triplicate pairs	119	10	109
quadruplicate pairs	22	0	22
quintuplicate pairs	1	0	1
sextuplicate pairs	3	0	3
total	***1432***	***110***	***1322***

Reasons for exclusion	*n*
Plate 6 high error rate	58
Mismatching pairs	3
Known under two ID codes	2
Sister took part too	1
Not northern European	9
Missing genotype calls on > 13 / 26 SNPs	38
One sample overlapped (not EU and mismatched)	-1
total n exclusions	***110***

Final sample overview	*n*
London cases	270
Utrecht cases	281
Vienna cases	97
total number of cases	*648*
London controls	447
Vienna controls	227
total number of controls	*674*
total n final	***1322***

Table 11: Overview of exclusions, and final sample for analyses

2.5. *SNP quality control*

2.5.1. Error rates

In total 26 SNPs were run on Sequenom. From the sample quality control it became clear that three SNPs had a high NTC error rate (above 10%, see Table 8, page 71); of these rs6355 and rs2383378 did not have elevated duplicate and triplicate error rates (<2%, see Table 10). The SNP rs4633 had a very high NTC

error rate (80%), as well as elevated duplicate and triplicate error rates (5% and 12.61% respectively, see Table 10). If the inflated error rate was caused by DNA contamination it would have been consisted across SNPs, since it is not this appears to be a marker specific problem related to the Sequenom panel design. Indeed the error rate of rs4633 can be explained by a shift in mass of the primer peak of rs12728678. The C allele of rs4633 is expected at 5,764 Dalton (Da), the primer of rs12728678 is expected at 5,797 Da (also see Figure 8, page 69). Mass spectra of NTCs show that this primer peak is missing where the C allele of rs4633 is called, indicating that the mass of this primer has turned out slightly lower than anticipated (see Figure 9, Figure 10 and Figure 11), and this explains the high NTC error rate of rs4633. Supportive of this assumption is the fact that the call of rs4633 in NTCs is always a C, and never a T allele.

The genotype call for rs4633 will thus be reliable in samples with good DNA quantity and quality, because after a successful iPlex Gold reaction no primer peaks will be present (also see paragraph 2.3.3, page 66). Apparently for the mismatching duplicate and triplicate samples the DNA quality or quantity was low on one of the samples resulting in an incomplete iPlex Gold reaction and left-over primer for rs12728678 which mistakenly was interpreted as the C allele of rs4633 (see Figure 9, Figure 10 and Figure 11). Since samples with obvious poor DNA quantity or quality (i.e. missing genotype calls on more than 13 out of 26 SNPs, see 2.4.3, page 76) will be excluded from analyses the genotype call of rs4633 will generally be reliable, but the SNP will nevertheless be excluded at this point. Rs4633 was one of the SNPs communicated to us personally prior to publication, but it was not part of the published associations to AN in the end (also see paragraph 2.2 Aim and outline, page 64), thus at this point in time there would be no reason to repeat the laboratory work on this particular SNP.

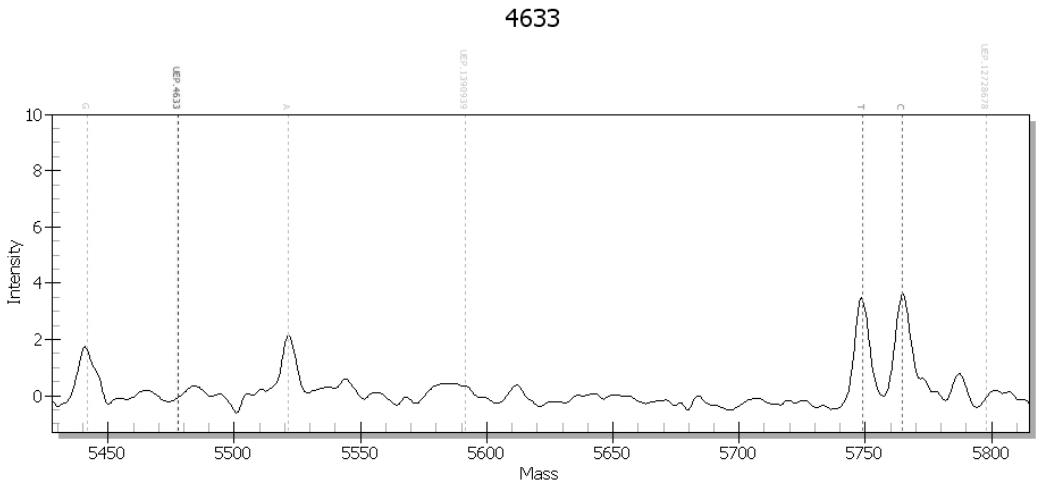

Figure 9: Mass spectrum of rs4633 - control DNA sample, well A2, Sequenom plate 1
This is the spectrum of a London control DNA sample heterozygote for rs4633; a peak is visible at both 5,748 Dalton (Da) (T allele) and 5,764 Da (C allele). There is no peak at 5,477 Da (primer of rs4633) nor at 5,797 Da (primer of rs12728678), indicating that the iPlex Gold reaction worked well.

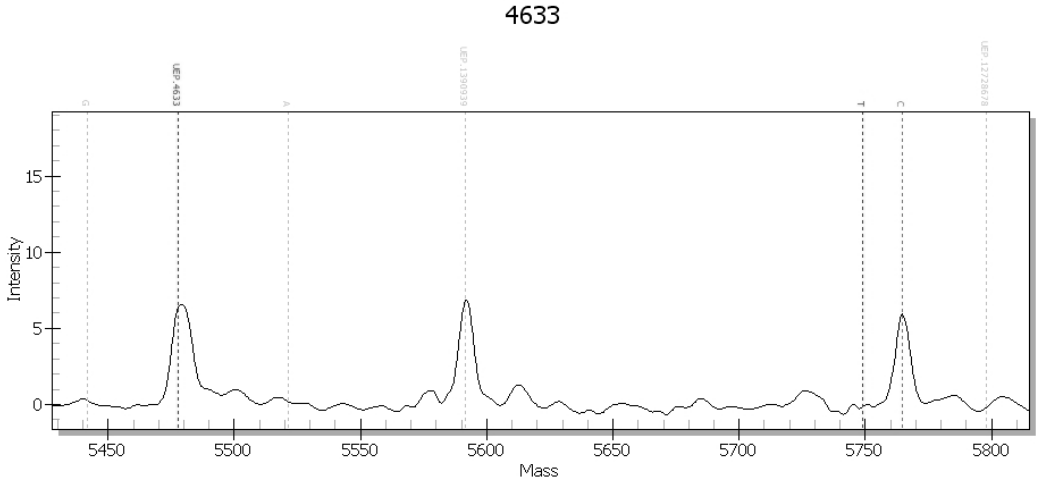

Figure 10: Mass spectrum of rs4633 – NTC, well L14, Sequenom plate 1
In this NTC of plate 1 there is an unexpected peak at 5,764 Da – resulting in the call of a C allele for rs4633. Slightly heavier than the C allele is the primer for rs12728678 at 5,797 Da (also see Figure 8, page 69) – this peak is missing in this graph (most right grey line). The primer of rs12728678 appears to have turned out slightly lower than anticipated, causing the primer peak to be mistakenly interpreted as the C allele of rs4633 (also see Figure 11). The primer peak for rs4633 is visible on the left side of the graph at 5,477 Da – as expected in an NTC.

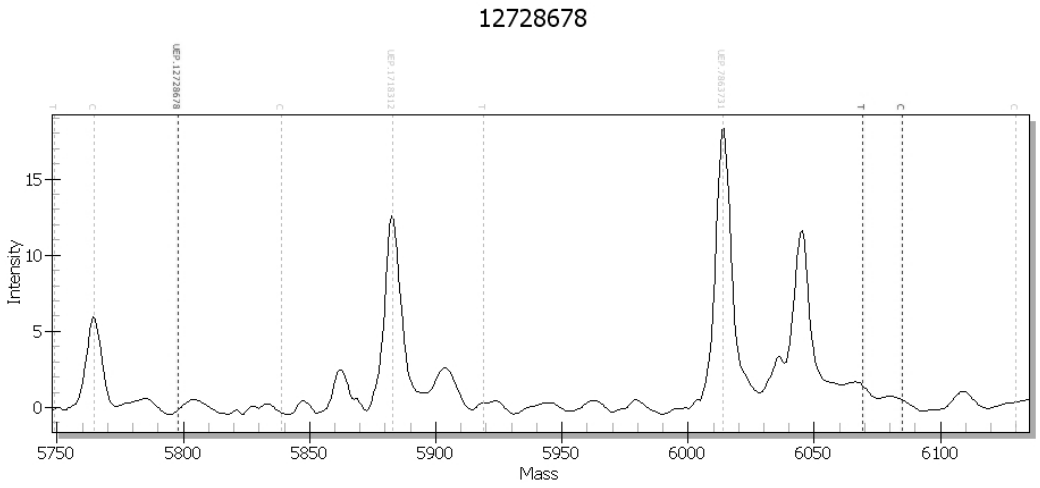

Figure 11: Missing primer peak for rs12728678 – NTC well L14 Sequenom plate 1
An alternative explanation for the called C allele of rs4633 and the missing primer peak for rs12728678 is that the NTC was contaminated with DNA. If this were the case than we would expect to see a genotype call for rs12728678, which is not the case (see the two dotted lines most right, there are no peaks). This figure shows that both the primer peak and the genotype peaks of rs12728678 are missing, supportive of the assumption that the mass of the primer of rs12728678 turned out slightly lower than anticipated causing it to be mistakenly interpreted as the C allele of rs4633.

2.5.2. Missingness

Missingness of genotype calls per SNP was calculated on samples passing sample quality control (n= 1,322, see Table 11) by dividing the number of missing genotypes by the number of samples. Six SNPs had missing genotype calls on more than 10% of the samples, this concerned three SNPs published as a haplotype associated with AN by Pinheiro *et al* (Pinheiro et al., 2010) (in ***italic***, see Table 12). The mean missingness resulted from missingness in the case sample and the Vienna control sample; apart from rs17210001 (bottom row, Table 12) none of the SNPs had high missingness in the London control samples. In order to check whether missingness was related to case/control status, country of origin, DNA source and/or DNA extraction method mean missingness (across all 26 SNPs) was calculated per sample (see Table 13). The London control sample (from blood) had the lowest mean missingness (3%), and the Utrecht case sample had the highest mean missingness (15%) but there is no apparent relationship between caseness and missingness (see Table 13). DNA from cheek swab samples extracted by the in-house method of Freeman *et al* (Freeman et

al., 1997,Freeman et al., 2003) did not have an apparent higher missingness compared to DNA extracted from blood in this sample. No SNPs will be excluded from analyses based on missingness, however the results of the haplotypes should be interpreted with care.

Missingness per SNP in percentages

rs#	*All cases (n= 648)*	*London controls (n= 447)*	*Vienna controls (n= 227)*	*All samples (n= 1322)*
4633	0%	2%	0%	1%
4680	**4%**	**1%**	**7%**	**4%**
6355	0%	2%	1%	1%
363221	1%	0%	0%	0%
363251	*14%*	*0%*	*19%*	*10%*
709611	0%	0%	0%	0%
987778	**1%**	**0%**	**2%**	**1%**
1055663	1%	1%	0%	1%
1126758	0%	0%	0%	0%
1390939	5%	2%	4%	4%
1718312	**9%**	**0%**	**11%**	**6%**
1801153	***35%***	***0%***	***44%***	***25%***
2020933	3%	0%	0%	2%
2173114	***69%***	***0%***	***55%***	***43%***
2383378	**2%**	**0%**	**2%**	**1%**
2917928	*22%*	*0%*	*33%*	*17%*
3808955	0%	0%	1%	1%
7820517	**0%**	**0%**	**0%**	**0%**
7836907	***13%***	***0%***	***26%***	***11%***
7863731	1%	2%	0%	1%
10444117	2%	0%	1%	1%
11264262	0%	0%	0%	0%
11564771	30%	0%	38%	*21%*
12728678	2%	1%	0%	1%
12831013	**3%**	**0%**	**1%**	**2%**
17210001	*77%*	*71%*	*64%*	*73%*
mean %	*11%*	*0%*	*12%*	*9%*

Table 12: Missingness per SNP in cases and controls
Six SNPs have a missingness above 10% (in *italic*), they have a high missingness in cases and in Vienna controls but not in London controls. SNPs shaded in grey were published in relation to AN by Brandys *et al* (in press) and by Wang *et al* (Wang et al., 2011), SNPs in bold were associated with AN as haplotypes by Pinheiro *et al* (Pinheiro et al., 2010). Rs4633 (top row, in grey) was excluded from analyses due to a design problem with the Sequenom panel, none of the other SNPs will be excluded from analyses but results of the haplotypes should be interpreted with care.

Missingness per country, DNA source, and extraction method

Sample	*Location*	*Case:Control*	*Source*	*DNA Extraction*	*Mean missingness*
1	London	case	blood	Nucleon BACC II kits by GE Life Sciences	6%
2	London	case	blood	in house variation of phenol/chloroform	10%
3	London	case	cheek swab	in house variation of phenol/chloroform	13%
4	Vienna	case	cheek swab	in house variation of phenol/chloroform	14%
5	Utrecht	case	blood blood	salting out method Genecatcher kits by Invitrogen	15%
6	London	control	blood	in house variation of phenol/chloroform	3%
7	Vienna	control	cheek swab	in house variation of phenol/chloroform	12%

Table 13: Missingness per country, case/control status, DNA source, and DNA extraction method (n= 26 SNPs)
Control samples are shaded in grey. The London control sample had the lowest mean missingness (3%), the Utrecht case sample had the highest mean missingness (15%) but there is no apparent relationship between caseness and missingness (see Table 12 for details per SNP). DNA extracted from cheek swab samples does not have an apparent higher missingness compared to DNA extracted from blood.

2.5.3. Reference population, and Hardy Weinberg Equilibrium

The genotype frequencies were compared to genotypes frequencies of a reference population for Europe, namely the HapMap reference population CEU: Utah residents with Northern and Western European ancestry (HapMap, 2003). The genotype frequencies match ± 10% (control sample only (n=674), maximum genotype frequency difference is 0.095, see Table 15). The details of the unpublished SNPs are presented in the Appendix (see Table 52, page 231). A final quality control measure is a test of the Hardy Weinberg Equilibrium (HWE); the HWE assumes that allele and genotype frequencies remain constant in a normal population (i.e. a normally sized population in which there is random mating). True deviations from HWE are very rare, thus in genetic studies it is used as a technical quality control measure i.e. significant deviations from HWE would indicate genotyping was erroneous. Since a true disease allele could theoretically cause a deviation from HWE it is calculated in controls only.

Allele frequencies were calculated from observed genotype frequencies (i.e. the frequency of A and B was calculated from the observed AA, AB, and BB frequencies), expected genotype frequencies were calculated based on allele

frequencies, and the difference between observed and expected genotype frequencies was tested by a chi-square test with one degree of freedom (see Table 14 for calculation details). One of the published SNPs is borderline significant for the HWE test (p=0.05, rs7820517, in ***bold*** Table 15, published as part of a haplotype (Pinheiro et al., 2010)), but since its genotype frequencies match the HapMap frequencies it will not be excluded from analyses. The results of this haplotype should however be interpreted with care.

HWE calculation	
Allele frequency	*number of alleles divided by the total number of alleles*
Expected genotype frequency	*allele frequency^2 * number of genotypes*
Chi-square	*difference between the observed and expected frequencies*
	1 degree of freedom

Table 14: HWE calculation details
The table gives the calculation details for the two homozygote genotypes. The expected heterozygote genotype frequency is calculated by multiplying the two allele frequencies squared, followed by multiplying it by the total number genotypes (i.e. individuals) in the sample.

SNP rs#	*HWE p value*	*HapMap CEU homozygote*	*Control sample homozygote*	*Difference*	*HapMap CEU heterozygote*	*Control sample heterozygote*	*Difference*
rs987778	0.51	0.000	0.000	0.000	0.107	0.049	0.058
rs4680	0.57	0.292	0.293	0.001	0.460	0.486	0.026
rs1801153	0.29	0.619	0.625	0.006	0.319	0.340	0.021
rs2383378	0.57	0.381	0.374	0.007	0.451	0.484	0.033
rs7836907	0.22	0.788	0.765	0.023	0.204	0.226	0.022
rs12831013	0.32	0.900	0.925	0.025	0.100	0.075	0.025
rs1718312	0.76	0.460	0.403	0.057	0.416	0.459	0.043
rs7820517	***0.05***	0.763	0.674	0.089	0.220	0.307	0.087
rs2173114	0.22	0.167	0.262	0.095	0.567	0.474	0.093

Table 15: HWE and HapMap frequencies of the published SNPs
SNPs shaded in grey were published by Brandys *et al* (in press) and by Wang *et al* (Wang et al., 2011), the other SNPs were part of the haplotypes published by Pinheiro *et al* (Pinheiro et al., 2010). Details of the unpublished SNPs are presented in the Appendix (see Table 52, page 231). One SNP is suggestive of violating the HWE (upper row, in *italic*), but its genotype frequencies match the HapMap frequencies ± 10%. No SNPs will be excluded based on HWE or HapMap frequencies, but the results of the haplotypes should be interpreted with care.

2.5.4. Overview of SNP exclusions

Since only nine of the initially 26 selected SNPs were published only the published SNP results will be discussed in the chapter (also see paragraph 2.2

Aim and outline, page 64), the other SNPs will be presented in the Appendix (see Table 53 to Table 59, page 232). None of the nine published SNPs will be excluded from analyses based on error rates, missingness, HWE, or HapMap frequencies, but the results of all three haplotypes should be interpreted with care since all haplotypes contain one or more SNPs with either a high missingness (above 10%, see Table 12, in ***italic***) or a SNP borderline significant for violation of the HWE (rs7820517, see Table 15).

2.5.5. Statistical methods

Case-control and within-case genotypic and allelic associations were tested by maximum-likelihood inference (Unphased software, version 3.4.1 (Dudbridge, 2008)). The Unphased method compares the probability of the observed distribution to occur under the hypothesis of true association versus the probability of the observed distribution to occur under the null-hypothesis. The case-control results are practically equal to a standard chi-square test, but Unphased, in contrast to chi-square, can still accurately test for association in case the allele counts are very low (below five counts). Haplotype analyses were also performed by maximum-likelihood inference (Unphased software, version 3.4.1 (Dudbridge, 2008)). The window size was fixed at three SNPs, i.e. only the specific combination of the three SNP haplotype published by Pinheiro *et al* (Pinheiro et al., 2010) was tested. Individuals with missing genotypes were excluded list-wise per haplotype. No frequency threshold was applied, i.e. if a haplotype combination was observed in the sample it was part of the analyses even if it was very rare.

Power calculations were conducted using the Genetic Power Calculator by Purcell *et al* ((Purcell et al., 2003)) with the following variables: 367 cases and a control:case ratio of 2, prevalence AN 0.6% (Jacobi et al., 2004b,Hudson et al., 2007,Swanson et al., 2011), genotype relative risks based on an additive genetic model (i.e. the risk of the homozygote genotype is the squared risk of the

heterozygote genotype), risk allele frequency was based on the control allele frequency (n= 674), α = 0.05, and power = 80%). Lastly, multiple testing was taken into account by using a Bonferroni correction of α = 0.05 divided by the number of tests.

2.6. Data description

Individual phenotype data were available for cases only, summary age and gender data of controls were presented in paragraph 2.3.1 *Sample collection*, page 65. All cases were female apart from one; the male case was part of the Utrecht case sample. The male case was slightly younger than average (18 vs 26 years old), his current and lowest adult lifetime body mass index (BMI) were close to the sample mean, and his highest adult lifetime BMI was slightly lower than average (18.5 vs 21.9) (age of onset was missing).

2.6.1. Age

Age was missing for 265 out of 648 cases, it ranged from 13 to 64 with a mean of 26 years old (n=383, stdev=10.7, see Figure 12).

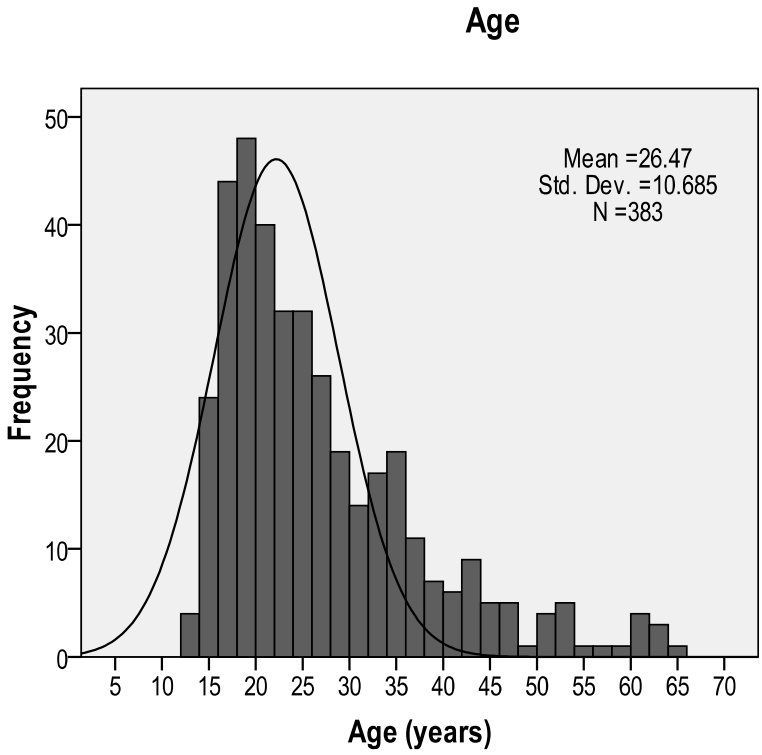

Figure 12: Distribution of age - AN cases

2.6.2. Age of onset

Age of AN onset was missing for most cases (405 out of 648), it ranged from 10 to 40 with a mean of 16 years old (n=243, stdev=3.8, see Figure 13).

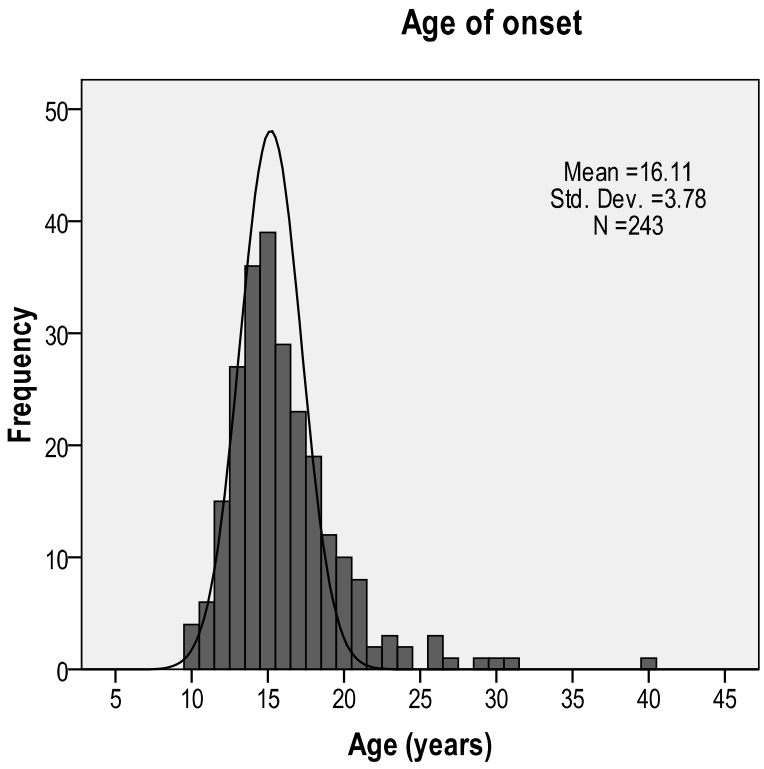

Figure 13: Distribution of age of onset - AN cases

2.6.3. Anorexia Nervosa subtype

For 153 cases (24%) information on subtype was missing. There were 296 (46%) cases of the restricting subtype and 199 (31%) cases of the binge/purge subtype. AN cases of all subtypes were analysed as a single group.

2.6.4. Current BMI

Current BMI was missing for 136 out of 648 cases, it ranged from 9.4 to 33.2 with a mean of 17.6 (n=512, stdev=2.8, see Figure 14).

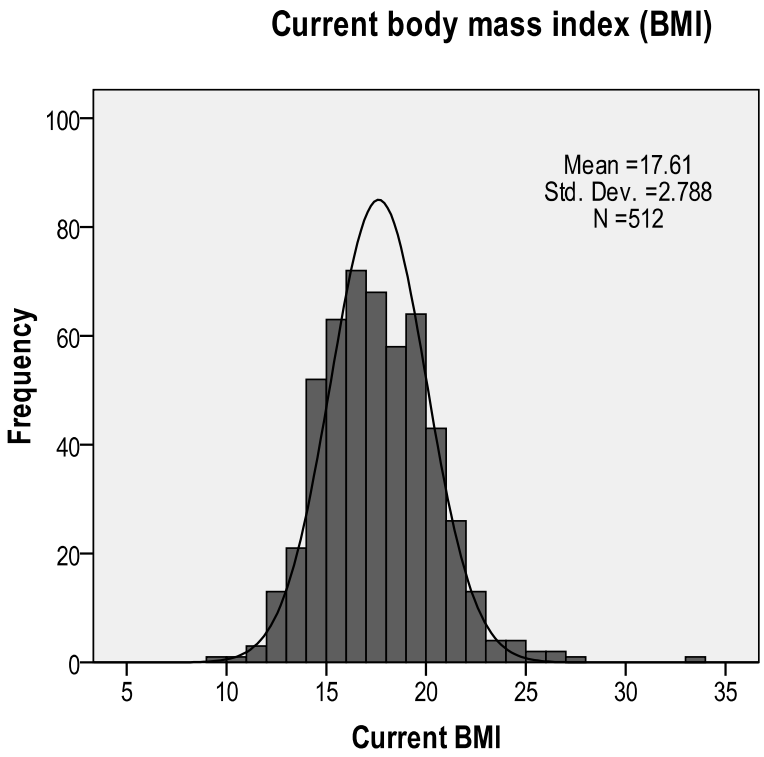

Figure 14: Distribution of current BMI - AN cases

2.6.5. Lowest adult lifetime BMI

For four cases their reported lowest adult lifetime BMI (i.e. lowest BMI) was higher than their current BMI and these lowest BMI data points were thus excluded. In total 146 out of 648 lowest BMI data points were missing. Lowest BMI ranged from 8.2 to 20.1 with a mean of 14.5 (n=502, stdev= 2.2, see Figure 15). Note: 36 cases reported a lowest adult lifetime BMI above 17.5 (one of the diagnostic criteria for AN); for which they, strictly speaking, should be excluded. However, because lowest adult lifetime BMI was self-reported, and diagnosis was made by structured clinical interviews based on DSM-IV (also see paragraph 2.3.1 *Sample collection*, page 65) cases were not excluded.

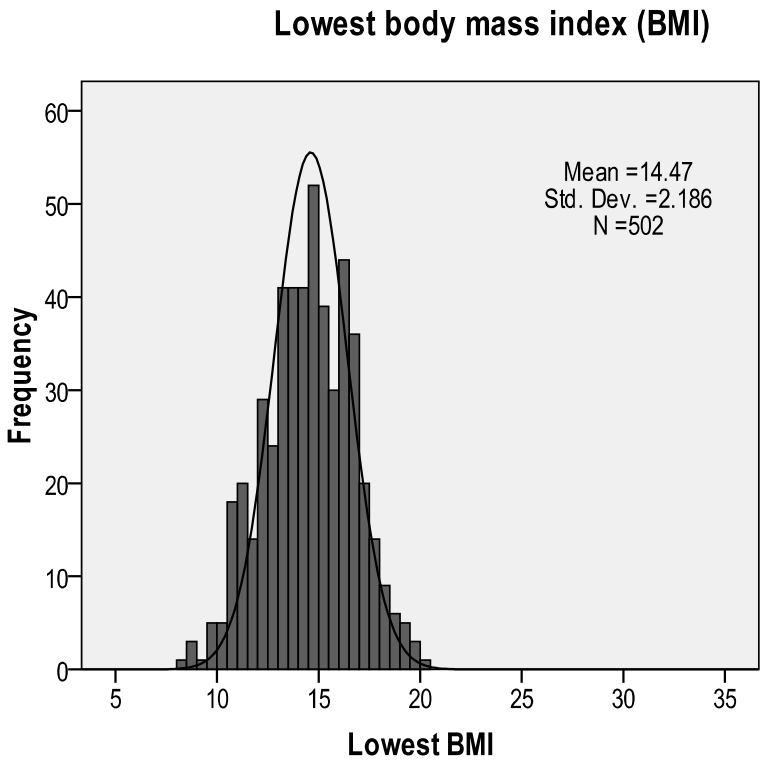

Figure 15: Distribution of lowest BMI - AN cases

2.6.6. Highest adult lifetime BMI

For five cases their highest adult lifetime BMI (i.e. highest BMI) was lower than their current BMI and these highest BMI data points were thus excluded from analyses. In total 205 out of 648 highest BMI data points were missing. Highest BMI ranged from 14.5 to 46.2 with a mean of 21.9 (n=443, stdev=3.5, see Figure 16).

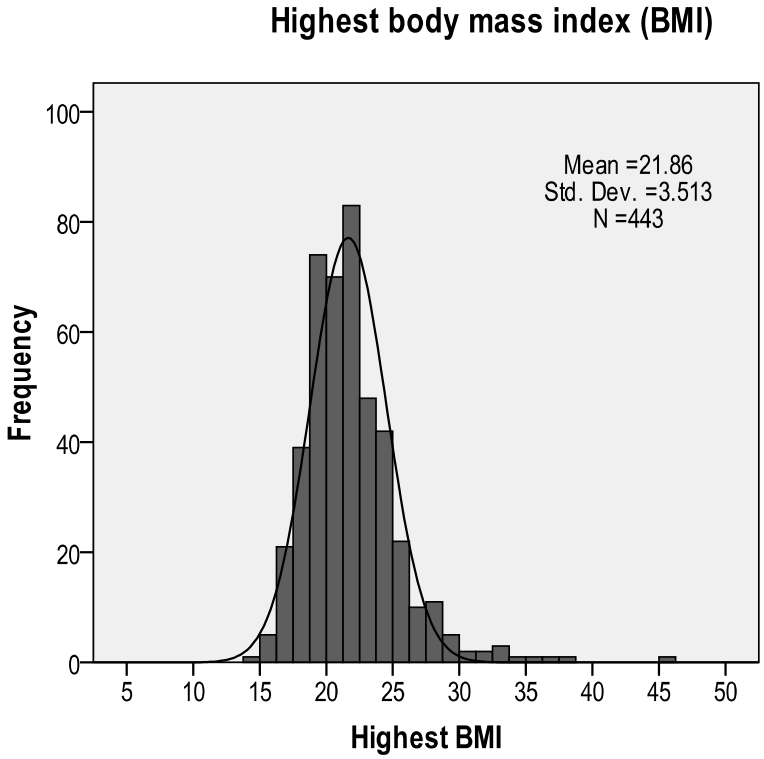

Figure 16: Distribution of highest BMI - AN cases

2.7. Results case-control analyses

2.7.1. Genotypic and allelic association

Of the SNPs from the published haplotypes by Pinheiro *et al* (Pinheiro et al., 2010) one SNP was suggestively associated with AN (rs1801153, p= 0.08, see Table 16). Note: they were published as a haplotype association with AN, not as single SNPs (also see paragraph 2.2 Aim and outline, page 64). When taking multiple testing into account none of the SNPs were significantly associated; when testing this many SNPs an association of this significance would be expected by chance. The top SNP published by Wang *et al* (Wang et al., 2011) was not associated with AN in the current sample (p= 0.59, see Table 17), however the current sample was underpowered to pick up effects with odds ratios smaller than 1.3 for heterozygote genotypes (1.69 for homozygote genotypes, also see paragraph 2.5.5 *Statistical methods*, page 84). The results

of the unpublished SNPs are presented in the Appendix (see Table 53 and Table 54, page 232).

Pinheiro et al		*Allele 1*	*Heterozygote*	*Allele 2*	*Missing*	*p genotype*	*p allele*
rs12831013	Case	329 (92%)	29 (8%)	1 (0%)	2%	0.33	0.51
	Control	621 (93%)	50 (7%)	0 (0%)	0%		
rs1718312	Case	34 (10%)	168 (49%)	143 (41%)	6%	0.19	0.27
	Control	89 (14%)	297 (46%)	261 (40%)	4%		
rs1801153	*Case*	*185 (68%)*	*73 (27%)*	*14 (5%)*	*26%*	***0.08***	***0.35***
	Control	*358 (62%)*	*195 (34%)*	*20 (3%)*	*15%*		
rs2173114	Case	36 (21%)	91 (53%)	46 (27%)	53%	0.31	0.36
	Control	144 (26%)	260 (47%)	145 (26%)	19%		
rs7820517	Case	255 (69%)	106 (29%)	6 (2%)	0%	0.76	0.48
	Control	454 (67%)	207 (31%)	13 (2%)	0%		
rs7836907	Case	260 (77%)	72 (21%)	5 (1%)	8%	0.73	0.95
	Control	471 (76%)	139 (23%)	6 (1%)	9%		
rs987778	Case	340 (95%)	18 (5%)	1 (0%)	2%	0.35	0.67
	Control	636 (95%)	33 (5%)	0 (0%)	1%		

Table 16: Results case control analyses - Pinheiro *et al*
One SNP of the top haplotype SNPs (Pinheiro et al., 2010) was suggestive associated with AN (genotype p value=0.08, in ***bold***), however when taking multiple testing into account none of the SNPs were significantly associated with AN. Some SNPs had a high percentage of missingness: see Table 12, page 81 for details on missingness.

Wang et al		*CC*	*CA*	*AA*	*Missing*	*p genotype*	*p allele*
rs2383378	Case	131 (36%)	174 (48%)	56 (16%)	2%	0.84	0.59
	Control	250 (37%)	323 (48%)	95 (14%)	1%		

Table 17: Results case control analyses - Wang *et al*
The top SNP from Wang *et al* (Wang et al., 2011) was not associated with AN in the current sample, however the current sample was underpowered to detect small effects (OR < 1.3).

Brandys *et al* published a negative result (Brandys *et al*, Anorexia nervosa and the Val158Met polymorphism of the COMT gene: meta-analysis and new data, in press), and the results of the current sample supported this (p= 0.27, see Table 18). The odds ratio (OR) of the current sample was comparable to the results of the Utrecht cohort published by Brandys *et al* (1.11 vs 1.14; see Table 18 vs Table 5, page 63), and it was slightly higher than the mean OR of the published meta-analysis (OR 1.03, see Table 5, page 63). Note: the Utrecht cases were part of both the current study and the published study by Brandys *et al*, but the Utrecht

samples were not part of the results in Table 18 since they were only part of the within-case study (also see paragraph 2.2 Aim and outline, page 64). Brandys *et al* presented a suggestive dominant effect of the Met-allele for the Utrecht cohort (p= 0.03, see Table 5, page 63) which was not replicated in their meta-analysis (p= 0.18), the current sample supports the meta-analysis results; there is no sign of a dominant effect (see Figure 17).

Brandys et al		*AA*	*AG*	*GG*	*Missing*	*p genotype*	*p allele*
rs4680	Case	89 (26%)	176 (51%)	82 (24%)	5%	0.47	0.27
	Control	191 (29%)	317 (49%)	144 (22%)	3%		

OR= 1.11 (0.92 - 1.33)

Table 18: Results case control analyses - Brandys *et al*
The results of the current study were supportive of the results published by Brandys *et al*; rs4680 was not associated with AN (Brandys *et al*, Anorexia nervosa and the Val158Met polymorphism of the COMT gene: meta-analysis and new data, in press). The A allele is the Methionine (Met) allele.

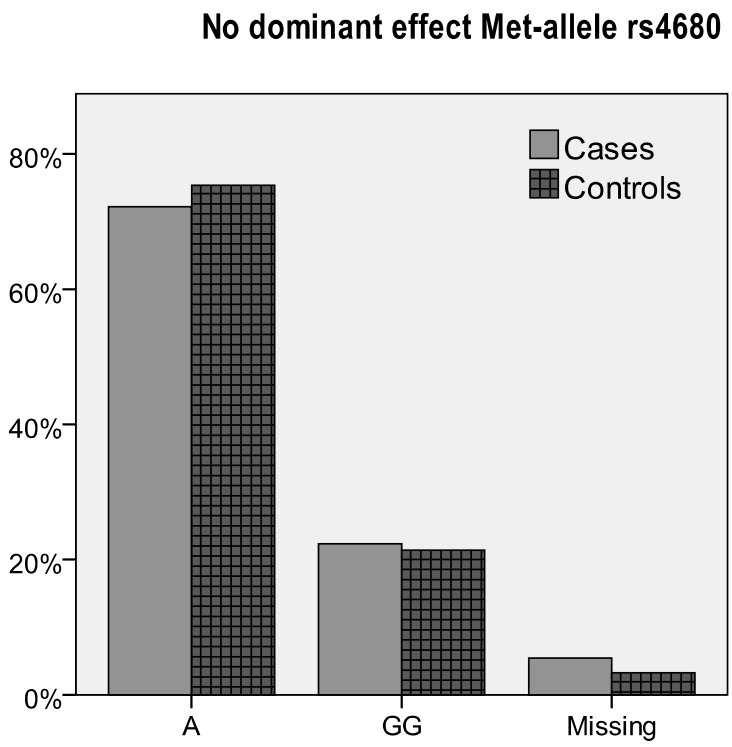

Figure 17: No dominant effect Met-allele (rs4680) COMT gene
In the Utrecht cohort of Brandys *et al* the Met-allele (A-allele) was suggestively

associated using a dominant genetic model, i.e. the presence of one or two A alleles (AA or AG, indicated by A) was significantly associated with AN compared to no A alleles present (GG) (p= 0.03 Table 5, page 63). In the current sample there was no sign of a possible dominant genetic effect; the Met-allele (A allele) was not more prevalent among cases than among controls (77% vs 78%).

2.7.2. Haplotypic association

None of the haplotype combinations showed a sign of association with AN in the current sample, p values of overall associations were 0.37, 0.68, and 0.44 respectively (see Table 19 to Table 21).

Haplotype	*Case (n)*	*Control (n)*	*OR*	*95% low*	*95% high*	*p value*
C-G-G	194	417	1.00	1.00	1.00	0.68
C-A-G	0	3	0.00	0.00	0.00	0.29
C-A-C	0	2	0.00	0.00	0.00	0.63
T-G-G	249	490	1.09	0.87	1.37	0.24
T-G-C	0	1	0.00	0.00	0.00	0.47
T-A-G	74	185	0.86	0.63	1.18	0.15
T-A-C	*27*	*44*	*1.32*	*0.79*	*2.21*	*0.34*
n alleles	*544*	*1142*				

Test of overall association: chisq= 6.5, df= 6, p value= 0.37

Table 19: Result haplotype rank 1 - Pinheiro *et al*
Haplotype combinations are in the order (rs1801153 - rs1718312 - rs12831013). The haplotype combination with the highest odds ratio (OR) is indicated in *italic*. There was no sign of association between any of the haplotype combinations and AN.

Haplotype	*Case (n)*	*Control (n)*	*OR*	*95% low*	*95% high*	*p value*
C-C-T	94	327	1.00	1.00	1.00	0.39
C-C-A	59	198	1.04	0.72	1.50	0.69
C-G-T	183	544	1.17	0.88	1.55	0.28
A-C-T	*8*	*22*	*1.26*	*0.54*	*2.93*	*0.83*
A-G-T	0	3	0.00	0.00	0.00	0.50

Test of overall association: chisq= 2.3, df= 4, p value= 0.68

Table 20: Result haplotype rank 9 - Pinheiro *et al*
Haplotype combinations are in the order (rs987778 - rs2173114 - rs7820517). The haplotype combination with the highest odds ratio (OR) is indicated in *italic*. There was no sign of association between any of the haplotype combinations and AN.

Haplotype	*Case (n)*	*Control (n)*	*OR*	*95% low*	*95% high*	*p value*
C-T-A	96	304	1.00	1.00	1.00	0.90
C-T-T	8	46	0.57	0.25	1.31	0.18
C-A-A	26	109	0.75	0.45	1.23	0.20
C-A-T	33	89	1.19	0.75	1.90	0.44
G-T-A	182	547	1.06	0.79	1.41	0.37
G-T-T	*1*	*3*	*1.27*	*0.12*	*13.87*	*0.88*

Test of overall association: chisq= 4.8, df= 5, p value= 0.44

Table 21: Results haplotype rank 17 - Pinheiro *et al*
Haplotype combinations are in the order (rs2173114 - rs7820517 - rs7836907). The haplotype combination with the highest odds ratio (OR) is indicated in *italic*. There was no sign of association between any of the haplotype combinations and AN.

2.8. Results within-case analyses – lowest BMI

2.8.1. Genotypic and allelic association

Of the SNPs from the published haplotypes by Pinheiro *et al* (Pinheiro et al., 2010) one SNP was suggestively associated with case-only lowest adult lifetime BMI (rs1718312, p= 0.02, see Table 22). Note: this is not the same SNP suggestively associated with AN in the case control analyses (see Table 16, page 91). However, when taking multiple testing into account none of the SNPs were significantly associated (Bonferroni threshold is α = 0.05 divided by nine SNPs is 0.006). The results of the SNPs from the haplotypes needed to be interpreted with care since several of the SNPs had a high missingness or were suggestive of violating the HWE; however there were no problems with rs1718312 (see Table 12 and Table 15). The top SNP published by Wang *et al* (Wang et al., 2011) was also not associated with lowest BMI in the current sample (p= 0.76), nor was the SNP published by Brandys *et al* (p= 0.20) (see Table 22). The results of the unpublished SNPs are presented in the Appendix (see Table 55, page 234).

Results within-case analyses - lowest BMI

Pinheiro et al		*Allele 1*	*Heterozygote*	*Allele 2*	*n*	*Missing*	*p genotype*	*p allele*
rs12831013	*Case*	445 (91%)	41 (8%)	1 (0%)	487	3%	0.12	0.69
rs1718312	*Case*	*62 (13%)*	*218 (44%)*	*186 (38%)*	*466*	*6%*	***0.02***	***0.10***
rs1801153	*Case*	*220 (45%)*	*101 (21%)*	*17 (3%)*	*338*	*26%*	*0.75*	*0.84*
rs2173114	*Case*	39 (8%)	91 (19%)	44 (9%)	174	51%	0.38	0.43
rs7820517	*Case*	347 (71%)	141 (29%)	13 (3%)	501	1%	0.63	0.38
rs7836907	*Case*	336 (68%)	101 (21%)	5 (1%)	442	10%	0.11	0.14
rs987778	*Case*	473 (96%)	22 (4%)	1 (0%)	496	1%	0.44	0.46
Wang et al								
rs2383378	*Case*	181 (37%)	240 (49%)	74 (15%)	495	2%	0.92	0.76
Brandys et al								
rs4680	*Case*	132 (27%)	245 (50%)	102 (21%)	479	4%	0.42	0.20

Table 22: Results within-case analyses - lowest BMI
One SNP is suggestive of association (rs1718312, genotypic p= 0.02, in ***bold***), see Figure 18 for details. However, taking correction for multiple testing into account none of the SNPs were associated with AN (Bonferroni correction threshold is 0.006).

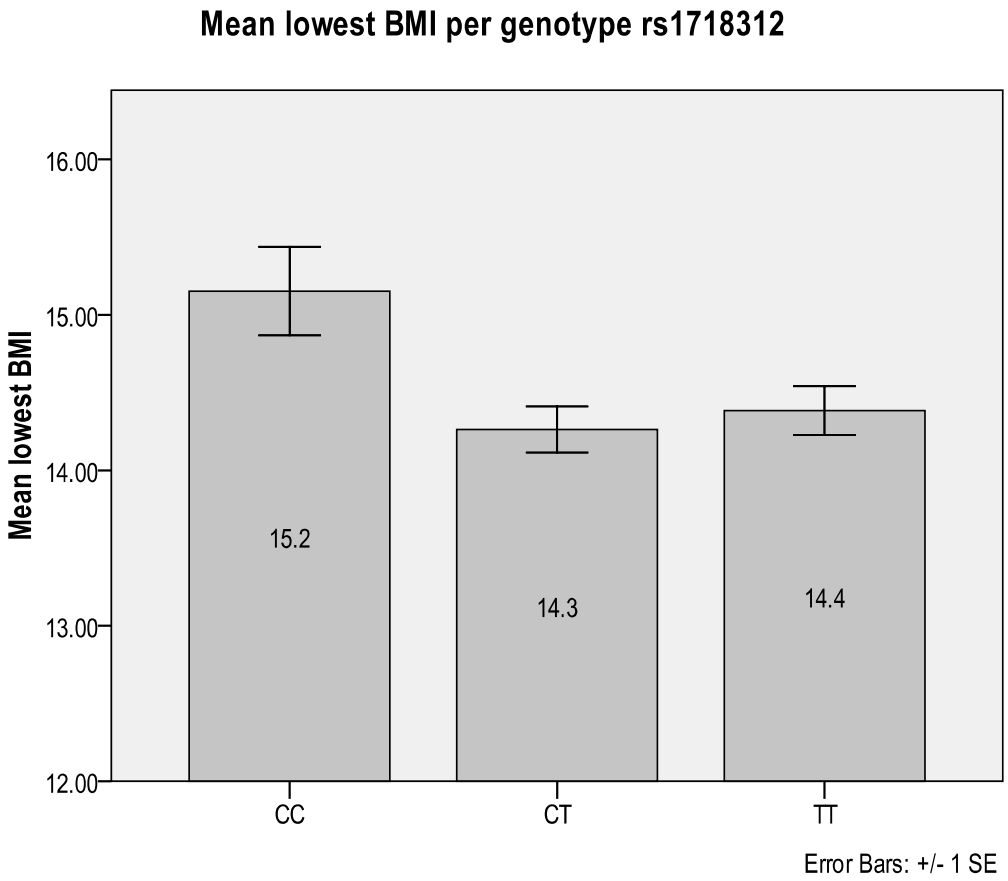

Figure 18: Suggestive association between rs1718312 and lowest BMI
The genotype of rs1718312 was suggestively associated with lowest BMI (p= 0.02, see Table 22), however when taking multiple testing into account there is no significant association; a difference like this would be expected by chance when testing nine SNPs for association. In this sample the individuals with a CC genotype had a higher lowest BMI (n= 78) compared to individuals with a CT or TT genotype (n= 269, and n= 244 respectively).

2.8.2. Haplotypic association

There is no sign of overall association of the haplotypes with case-only lowest BMI in the current sample; p values of overall associations were 0.47, 0.57, and 0.24 respectively. The haplotype combination C-A-T (haplotype rank 17 Pinheiro *et al*, see Table 2, page 61) was however suggestively associated (p= 0.03, see Table 23), but when taking multiple testing into account it is not significant; p values like this would be expected by chance when testing this many

associations. The results of the other haplotypes are presented in the Appendix (see Table 57 and Table 58, page 235).

Haplotype	*Case (n)*	*Frequency*	*p value*
C-T-A	101	29%	0.73
C-T-T	10	3%	0.39
C-A-A	22	6%	0.37
C-A-T	*34*	*10%*	*0.03*
G-T-A	178	51%	0.38
G-T-T	2	0%	0.71

Test of overall association: chisq= 6.7, df= 5, p value= 0.24

Table 23: Results within-case association - lowest BMI - haplotype rank 17 Pinheiro *et al*
The haplotype combination C-A-T (rs2173114 - rs7820517 - rs7836907) was suggestively associated with lowest BMI, however when taking multiple testing into account there is no significant association.

2.9. Results within-case analyses – highest BMI

2.9.1. Genotypic and allelic association

None of the SNPs showed a sign of association with case-only highest adult lifetime BMI (see Table 24), rs1801153 has the lowest p value (genotype p value= 0.12). The results of the unpublished SNPs are presented in the Appendix (see Table 56, page 235).

Results within-case analyses - highest BMI

Pinheiro et al		*Allele 1*	*Heterozygote*	*Allele 2*	*n*	*Missing*	*p genotype*	*p allele*
rs12831013	*Case*	387 (89%)	42 (10%)	1 (0%)	487	12%	0.46	0.93
rs1718312	Case	58 (13%)	189 (44%)	162 (37%)	466	15%	0.91	0.37
rs1801153	Case	193 (45%)	91 (21%)	14 (3%)	338	32%	0.12	0.80
rs2173114	*Case*	37 (9%)	76 (18%)	41 (9%)	174	54%	0.39	1.00
rs7820517	*Case*	308 (71%)	122 (28%)	13 (3%)	501	10%	0.57	0.92
rs7836907	*Case*	293 (68%)	89 (21%)	3 (1%)	442	19%	0.41	0.81
rs987778	*Case*	418 (97%)	19 (4%)	1 (0%)	496	10%	0.42	0.34
Wang et al								
rs2383378	*Case*	151 (35%)	221 (51%)	65 (15%)	495	11%	0.86	0.84
Brandys et al								
rs4680	*Case*	124 (29%)	216 (50%)	83 (19%)	479	13%	0.57	0.98

Table 24: Results within-case analyses - highest BMI
There is no sign of association of the SNPs with highest BMI.

2.9.2. Haplotypic association

There is no sign of overall association of haplotypes with case-only highest BMI in the current sample; p values of overall associations were 0.20, 0.27, and 0.87 respectively. The haplotype combination T-A-G (haplotype rank 1 Pinheiro *et al*, see Table 2, page 61) was however suggestively associated (p= 0.05, see Table 25), but when taking multiple testing into account it is not significant. The same holds true for the haplotype combination A-C-T (p= 0.04, haplotype rank 9 Pinheiro *et al*, see Table 26). The results of the other haplotype are presented in the Appendix (see Table 59, page 236).

Haplotype	*Case (n)*	*Frequency*	*p value*
C-G-G	222	37%	0.98
T-G-G	255	43%	0.12
T-A-G	*88*	*15%*	*0.05*
T-A-C	31	5%	0.80

Test of overall association: chisq= 4.6, df= 3, p value= 0.20

Table 25: Results within case association - highest BMI - haplotype rank 1 Pinheiro *et al*
The haplotype combination T-A-G (rs1801153 - rs1718312 - rs12831013) was suggestively associated with lowest BMI, however a p value like this would be expected by chance when testing this many associations.

Haplotype	*Case (n)*	*Frequency*	*p value*
C-C-T	87	28%	0.82
C-C-A	54	18%	0.42
C-G-T	158	52%	0.84
A-C-T	*7*	*2%*	*0.04*

Test of overall association: chisq= 3.8, df= 3, p value= 0.27

Table 26: Results within-case association - highest BMI - haplotype rank 9 Pinheiro *et al*
The haplotype combination A-C-T (rs987778 - rs2173114 - rs7820517) was suggestively associated with lowest BMI, however a p value like this would be expected by chance when testing this many associations.

2.10. Conclusion and discussion

2.10.1. Case-control analyses

The case control single nucleotide polymorphism (SNP) results indicate that one out of nine tested SNPs was suggestively associated with AN (rs1801153, genotypic p value = 0.08, allelic p value = 0.35, Table 16). When testing nine SNPs for association however, a result like this would be expected to arise by chance; it does not pass a Bonferroni correction for multiple testing. Moreover, this SNP had not been associated with AN as a single SNP, but as part of a haplotype (also see Table 2) (Pinheiro et al., 2010).

The top SNP found to be associated with AN in the first genome-wide association (GWA) study of AN by Wang *et al* (rs2383378, (Wang et al., 2011)) did not replicate in the current sample (genotypic p value = 0.84, allelic p value = 0.59, Table 17), however the current sample was underpowered to detect variants with small effect sizes (odds ratio below 1.5 for heterozygote genotypes, and below 2.25 for homozygote genotypes - given a minor allele frequency of 0.38 and a disease prevalence of 0.6%). In hindsight, I have to agree with the – somewhat uncomfortable – conclusion of Kim *et al* (Kim et al., 2011); that underpowered studies are not worth undertaking. There is (now) a large body of evidence indicating that common genetic risk factors underlying complex disorders such as EDs probably have a very small effect, somewhere the region of 1.1 to 1.2 (Cichon et al., 2009,Visscher et al., 2011), however – to my defence – this evidence was only unfolding as I was already conducting my laboratory work. The genotype data of this study could be used however in future meta-analyses. For this particular SNP (rs2383378) unfortunately there was an inflated negative control error rate; 59% of negative controls (which contain no DNA) had a genotype call for this SNP (Table 8, page 71), indicative of (technical) erroneous genotype calling. If the negative control rate had been inflated for all SNPs it

would be an indication of DNA contamination (also see paragraph 2.4 *Sample quality control*, page 70), which was not the case. The duplicate and triplicate error rates were normal for this SNP; 2% and 0.84% respectively (221 samples were run in duplicate, and 119 samples were run in triplicate, for quality control purposes; the error rate was calculated by dividing the number of mismatching duplicates/triplicates by the number of possible mismatches). Furthermore, the genotype distribution of this SNP matched the distribution of the reference population, and it did not violate the Hardy Weinberg Equilibrium (also see paragraph 2.5.3 *Reference population, and Hardy Weinberg Equilibrium*, page 82), hence it was not excluded from analyses in this chapter. I nevertheless found the inflated negative error rate worrying, and I would want to repeat the laboratory work prior to any meta-analyses. I did also genotype this particular SNP with a different technique; a *TaqMan* technique (McGuigan and Ralston, 2002), which is based on fluorescence rather than mass spectrometry. However the results were unusable, because the homozygote and heterozygote genotypes of rs2383378 did not cluster, indicative of technical genotyping problems with the *TaqMan* technique (data not shown). This is however a relatively common problem and repeating either the Sequenom or the *TaqMan* method would be needed before it could be concluded that this particular SNP is difficult to genotype.

The Val158Met polymorphism of the COMT gene has been associated with AN, though not unequivocally (Brandys et al, in press). Brandys *et al* conclude from their meta-analyses that the polymorphism is not associated with AN, and the results of this thesis support this conclusion; the odds ratio in the London and Vienna case and control sample was more equal to that of the Utrecht cohort (1.11 vs 1.14, Table 18), but the results were non-significant (genotypic p value 0.47, allelic p value 0.27). There was no sign of a possible dominant effect of the Met allele in this sample (also see Figure 17), which also supports the conclusion of Brandys *et al* (Brandys et al, in press).

The three selected haplotypes of the study by Pinheiro *et al* (Pinheiro et al., 2010) did not replicate in the current sample (p values of overall association were 0.37, 0.68, and 0.44 respectively). Haplotypes are however difficult to assess due to current technological limitations; actual (i.e. phased) haplotypes can not be measured directly, because raw genotyping data does not distinguish between maternally and paternally derived chromosomes. Thus when an individual is heterozygote for a SNP it can not be determined from which parental chromosome the allele came; the genotype data is said to be 'unphased'. The phase can not be measured directly, but is assigned using probablities of haplotypes based on LD and known haplotype structures (Browning and Browning, 2011). The problem with this method is however that minor inaccuracies in the estimation of haplotype frequencies can lead to spurious results (Curtis and Gurling, 2006,Curtis and Xu, 2007), and for this reason current genetic studies (like the genome-wide GCAN study for anorexia nervosa) tend to avoid haplotypes. As Browning and Browning point out (Browning and Browning, 2011), haplotype estimation can be improved by improving LD information through genotyping larger panels of individuals from different ethnicities, for which progress is being made as part of the 1000 Genomes project (1000 Genomes, 2010).

2.10.2. Within-case analyses

Lowest and highest adult lifetime body mass index (BMI) was tested for association with genetic variants previously (suggestively) associated with AN (Pinheiro et al., 2010,Wang et al., 2011,Brandys et al., in press). One out of nine tested SNPs was suggestively associated with lowest adult lifetime BMI; rs1718312 (genotypic p value = 0.02, allelic p value = 0.10 see Table 22). The CC genotype of this SNP had a higher lowest adult lifetime BMI compared to the CT and TT genotypes (15.2 versus 14.3 and 14.4, see Figure 18). A difference like this is however expected to arise by chance when testing nine SNPs for association;

the result does not pass a Bonferroni correction for multiple testing. Similarly one haplotype combination was suggestively associated with lowest adult lifetime BMI (p = 0.03, the combination C-A-T of haplotype rank 17 (Pinheiro et al., 2010), also see Table 4, page 62) however again when taking multiple testing into account it is not significant. Unfortunately Pinheiro *et al* (Pinheiro et al., 2010) only published the overall significance of the haplotypes instead of the significance of the specific haplotype combinations, hence it is unclear from the paper which haplotype combination (e.g. C-A-T) was most strongly associated to the phenotype in their sample. No SNPs were associated with highest adult lifetime BMI, but two haplotype combinations were suggestively associated; the combination T-A-G of haplotype rank 1 Pinheiro *et al* (Table 2, page 61, (Pinheiro et al., 2010)) (p = 0.05, see Table 25), and the combination A-C-T haplotype rank 9 Pinheiro *et al* (Table 2, page 61) (p = 0.04, see Table 26), however again the results do not pass a multiple testing correction.

The lack of association with lowest and highest adult lifetime BMI could indicate that these are not quantitative traits reflective of the underlying genetic liability for AN; the true underlying traits for EDs are unknown as yet. The non-significant results of this thesis support the results of the study by Root *et al* (Root et al., 2011), who also tested the association between genetic variants and quantitative traits relevant for AN (testing the same genetic variations and the same sample as (Pinheiro et al., 2010), who only performed case control analyses). Root *et al* found no significant results (after correction for multiple testing) for association with lowest adult lifetime BMI, nor for association with any of the other traits tested, including age at menarche, drive for thinness, body dissatisfaction, trait anxiety, concern over mistakes, and the anticipatory worry and pessimism versun uninhibited optimism subscale of the harm avoidance scale (Root et al., 2011).

The current study was underpowered to detect variants with small effects on lowest and highest adult lifetime BMI, similarly as to what was discussed for the results of the case control analyses (paragraph 2.10.1, page 99). If there were genetic risk variants underlying lowest and highest adult lifetime BMI in AN cases, and we assumed they had similar effect sizes as the genetic variants underlying current BMI (Day and Loos, 2011), a sample size of at least 100,000 individuals would be needed in order to have enough power to detect them (Day and Loos, 2011). For the studies of current BMI, a total of four waves of data collection have lead to the robust discovery of at least 50 genetic loci (reviewed by Day *et al* (Day and Loos, 2011)); in the fourth wave a meta-analysis of 46 studies (123,865 individuals) was conducted, of which the resulting top 42 SNPs were taken forward for replication in 125,931 additional individuals (Speliotes et al., 2010). Day and Loos show that the gene with the largest effect size (FTO) was discovered first, i.e. it was the lowest hanging fruit (Day and Loos, 2011). Per FTO risk allele BMI increases by 0.39 kg/m^2, which is equal to approximately 1.1 kilogram for someome 1.70m tall (represented by the black bar, see Figure 19 (Day and Loos, 2011)). The discovery of the genetic risk variant with the second most strong effect size (located near MC4R, the second bar from the left, see Figure 19) needed a much larger sample size, not only because its effect size was lower than FTO but also because its risk allele occurs less frequently in the population (Day and Loos, 2011). These results clearly demonstrate that the study I conducted was underpowered, and that much larger sample sizes would be needed.

[image unavailable in e-thesis]

Figure 19: The lowest hanging fruit (Day and Loos, 2011)
The genetic studies on body mass index (BMI) indicate that the gene with the largest effect on BMI (FTO, also see paragraph 1.1 Normal regulation of eating behaviour, page 20) was discovered first, i.e. it was the lowest hanging fruit. After four waves of data collection (n=125,931) 50 genetic risk variants have now robustly been associated with BMI. The effect size of the risk variants is expressed in the increase in body weight (gram) per risk allele for an individual 1.70m tall (the figure was obtained from reference (Day and Loos, 2011)).

2.10.3. Limitations and future directions

The most important limitations of the study presented in this chapter were the relatively small sample size (at least given the current knowledge on the genetic architecture of complex disorders, also see paragraph 1.3.4.3 *Undetected heritability and genetic architecture*, 52), and the fact that relatively few genetic variations were tested for association. Several studies now demonstrate that genetic variants cumulatively cause phenotypic variance in complex traits; Lango Allen *et al* show that hundreds of genetic variants are involved in the regulation of human height (Lango et al., 2010), Li *et al*, and Hebebrand *et al* present the cumulative effects of the genetic risk loci for BMI (Li et al., 2010,Hebebrand et al., 2010), and Davies *et al* predicted human intelligence scores based on the cumulative effect of genome-wide SNPs (Davies et al., 2011).

Since several genetic risk variants have now robustly been associated with BMI, and it presents a nice example of where the genetics of eating disorders may head towards in the future. Li *et al* illustrate how the mean BMI increases in a linear fashion with the number of genetic risk alleles (see Figure 20, (Li et al., 2010). On average the mean BMI increases with 0.15 kg/m^2 per additional genetic risk variant, and individuals with 17 or more genetic risk variants had a BMI that was 1.53 kg/m^2 greater than that of individuals with 6 or less genetic risk variants (Li et al., 2010). In a more recent paper Hebebrand *et al* update this calculation after more risk variants for BMI had been established, and they show that individuals with 38 or more genetic risk variants had a BMI 2.73 kg/m^2 greater than individuals with 21 or less genetic risk alleles (Hebebrand et al., 2010). This example illustrates the challenges of genetic research in eating disorders; it will be very difficult to assess a difference in the order of magnitude of "0.15kg/m^2" per genetic risk variant expressed in liability for AN, because – at least currently – there are no unequivocal measurements of severity of illness for eating disorders.

[image unavailable in e-thesis]

Figure 20: Increase in BMI per genetic risk variant (Li et al., 2010)
Mean BMI increases linearly with the number of genetic risk variant; on average 0.15 kg/m^2 per genetic risk variant.

Disordered eating in relation to body mass index

3. Disordered eating in relation to body mass index

This chapter will present the results of the Eating Disorder Inventory (EDI) questionnaire in a sample of 3,624 healthy female twins from the United Kingdom (UK). The mean age of the sample was 57 years, which is higher than most samples in the field of eating disorders; hence the literature introduction will provide some background on disordered eating in this age group. This chapter will not include genetic analyses, but it partly serves as an extensive introduction to the next chapter; Chapter 4 Genome-wide gene analyses (page 154), which will discuss the genome-wide genetic analyses of the EDI in this sample.

3.1. *Literature background*

The Eating Disorders Inventory (EDI) was developed by Garner, Olmsted, and Polivy in 1983 (Garner et al., 1983), and it aimed to measure behavioural and psychological traits in anorexia nervosa (AN) and bulimia nervosa (BN) (also see paragraph 1.2 Eating disorders, page 27, for the definition AN and BN, and their clinical presentation). The original version of the EDI comprised 64 items, but it was revised in 1991 to 91 items (EDI-2, Garner, 1991), and revised again in 2004 (EDI-3, Garner, 2004). In the last revision the items remained the same as for the EDI-2, but the internal structure was altered. In all EDI versions the respondents are asked whether an item applies to them “always”, “usually”, “often”, “sometimes”, “rarely”, or “never”. The 91 items are grouped in subscales, and the responses to the items can be added up per subscale. Three subscales specifically assess attitudes towards weight, body shape, and eating; the drive for thinness (DT), bulimia (B), and body dissatisfaction (BD) subscales; these are also known as the Eating Disorder Risk Scales. The other subscales assess more general psychological characteristics, such as: low self-esteem, personal alienation, interpersonal insecurity, interpersonal alienation,

interoceptive deficits, emotional dysregulation, perfectionism, ascetiscism, interoceptive awareness, and maturity fears. For the current study the general psychological characteristics were not assessed; only the items of the Eating Disorder Risk Scales; DT, B, and BD, were included in the self-report questionnaire that was sent to the participants (which will be discussed in more detail in paragraph 3.4 *Data description*, page 113). In the most recent version of the EDI, the EDI-3, the Eating Disorder Risk Scales were unchanged apart from the addition of one item from the EDI-2 'interoceptive awareness' scale to the 'bulimia' and the 'body dissatisfaction' scales (Garner, 2004), the exact questions and their corresponding subscales are presented in the Appendix (Table 60, page 237).

The EDI Eating Disorder Risk Scales DT, B, and BD, have been suggested as endophenotypes of eating disorders (EDs) (Stice and Shaw, 2002,Bulik et al., 2007a,Wilksch and Wade, 2009). An endophenotype basically is an 'intermediate' phenotype; it bridges between the complex clinal presentation and the underlying aetiology, i.e. it 'breaks' the disorder up into manageable 'pieces' which can be studied on their own; complex disorders can be disentangled in this way. The endophenotype concept has been suggested to lead to more straight forward, and more successful genetic analyses (Gottesman and Gould, 2003). Flint and Munafo however argue that endophenotypes may not have a more simple genetic architecture at all (Flint and Munafo, 2007). Moreover, Walters and Owen stress that, even though the endophenotype concept is very appealing, in reality endophenotypes may be state-dependent, or they may associate with the disease but not with the disease causing genes (Walters and Owen, 2007); stressing the need for very careful examination of endophenotypes. Several within- and between- disorder endophenotypes have been suggested for EDs (reviewed by Bulik *et al* (Bulik et al., 2007a), and by Treasure (Treasure, 2007)). Drive for thinness and body dissatisfaction have been studied more than the bulimia subscale of the EDI, and they have proven to be

among the most robust endophenotypes for EDs (Bulik et al., 2005,Bulik et al., 2007a,Stice and Shaw, 2002,Wilksch and Wade, 2009), though it should be noted that EDI is not the only method used to assess drive for thinness and body dissatisfaction (Tury et al., 2010). Note: only when specifically the drive for thinness, bulimia, and body dissatisfaction subscales of the EDI are discussed the abbreviations DT, B and BD will be used, in all other situations the abbreviations will not be used. Several studies have demonstrated that DT and BD are moderately heritable (Rutherford et al., 1993,Klump et al., 2000,Keski-Rahkonen et al., 2005) (Boraska et al, submitted), and DT and BD have been included as behavioural covariates in genetic analyses of EDs (Devlin et al., 2002,Root et al., 2011).

When interpreting the results of the EDI it is important to take note of the context in which the questionnaire was administered, because the scores may not represent the same thing in different samples, like samples from different cultures, different age groups, or different settings (e.g. clinical or community based). Podar and Allik conducted a large-scale cross-cultural comparison of the EDI literature in 2009 (Podar and Allik, 2009), their sample included 43,722 individuals from 25 countries across all continents. Out of the 310 samples Podar and Allik included a 136 were general population samples, 159 were clinical ED samples, and 15 were special samples (including ballet dancers, and specific psychiatric, and obesity samples) (Podar and Allik, 2009). The mean age was 21 years for individuals with AN, 25 years for individuals with BN, and 19.7 years for individuals from the general population (Podar and Allik, 2009). The mean body mass index (BMI) for the general population samples was 21.6 (with a standard deviation of 1.9) (Podar and Allik, 2009). Podar and Allik found, based on the mean scores of the subscales of the EDI, that the pairwise intercorrelation of the subscales suggested two main factors which accounted for 67% of the total variance; three personality scales loaded strongly on the first factor, and the Eating Disorder Risk scales DT, B, and BD, loaded strongly on the second factor

(Podar and Allik, 2009), which is in agreement with the intended distinction between eating behaviour attitudes and general psychological characteristics (Garner, 1983,Garner, 1991,Garner, 2004). This factor structure held true for both the general population as the clinical sample across cultures, indicating that the meaning of the scales is comparable between the general population and individuals with a clinical ED (Podar and Allik, 2009).

The sample I analysed in this chapter is of older age and higher BMI than the samples in the study by Podar and Allik (Podar and Allik, 2009). Even though EDs are typical for adolescent females (also see 1.2.1 *Prevalence and incidence*, page 29), disordered eating and weight and shape concerns do occur in older women as well (Lewis and Cachelin, 2001,Mangweth-Matzek et al., 2006,Bedford and Johnson, 2006,Slevec and Tiggemann, 2011). No differences in prevalence of body dissatisfaction were found between women of younger and older age groups (Webster and Tiggemann, 2003,Bedford and Johnson, 2006), but Webster and Tiggemann note that body dissatisfaction had less impact on the self-esteem of women who were older (Webster and Tiggemann, 2003).

The EDI scores tend to increase with body mass index (BMI) (Mangweth-Matzek et al., 2006,Packianathan et al., 2002). It should however be noted that this trend was observed in clinical samples, but not in the general population samples of the study by Podar and Allik (Podar and Allik, 2009); however the general population samples in the study by Podar and Allik were young, mean age 19.7 years (standard deviation 3.9), and had a low normal BMI, 21.6 (standard deviation 1.9). The mean BMI in the UK in 2007 for women was 26.8, and was generally higher in older age groups (Health Survey for England, 2007). Packianathan *et al* aimed to identify a normative range of EDI scores in an obese predominantly female UK sample with a mean age of 42 years (note: these were not patients seeking bariatric surgery) (Packianathan et al., 2002). They found

that most subscale scores were within the range of a normal population, except for DT and BD, which were elevated among obese individuals (Packianathan et al., 2002). Figure 21 shows the median raw scores of the EDI-2 of 100 unselected obese patients referred to an obesity clinic by their general practitioners, compared to a normative range of EDI scores in healthy college-aged females, and a normative range of EDI scores in eating disorder patients (see Figure 21). The obese sample scored significantly higher compared to the healthy women on the DT and on BD subscales; and 83% of the obese sample had a BD score above the normative range for eating disorders (half of the obese sample scored the maximum BD score) (Packianathan et al., 2002). This relationship between body dissatisfaction and BMI was also found in more recent studies on weight and shape concerns of older women (Mangweth-Matzek et al., 2006,Slevec and Tiggemann, 2011).

[image unavailable in e-thesis]

Figure 21: Normative range of the EDI (Packianathan et al., 2002)
In light grey the normative range of EDI scores in eating disorder patients is shown (labelled 'Eating Disorder norm'); in dark grey the normative range of EDI scores in healthy college-aged females is shown (labelled 'Female College Students norm'); and the black squares represent the median raw scores of the EDI subscales in the obese group (n = 100). The obese group scores higher on Drive for Thiness (DT), and Body Dissatisfaction (BD); the BD scores of most obese individuals are higher than the eatind disorder normative range (half of the obese samples scored the maximum BD score) (Packianathan et al., 2002).

3.2. Aim and outline

This aim of this chapter was to present the distribution and correlates of the drive for thinness (DT), bulimia (B), and body dissatisfaction (BD) eating disorder risk scales, as assessed by the Eating Disorder Inventory (EDI) (Garner, 2004), in a general population sample, with a specific focus on the relation with body mass index. The first part of this chapter tested the hypothesis that a higher current BMI was related to a higher DT, B, and BD score. The second part of this chapter aimed to test which BMI predicts DT, B, and BD scores best; namely current BMI, lowest adult lifetime BMI, highest adult lifetime BMI, or the combined BMI history of an individual. Note: any causal direction of these relationships, e.g. whether disordered eating preceded an increase in BMI or the other way around, could not be ascertained from this study.

3.3. Statistical methods

Analyses were performed using regression and multiple regression methods, and do not include twin modelling for heritability analysis. Twin relatedness was accounted for by multilevel analyses using Generalised Estimating Equations (GEE) in SPSS Statistics software version 17.0, with the twin family identifier as the subject effect and an indication of proband and non-proband as within-subject effect. Variables were entered into the model by forced entry. Cases with missing values were excluded list-wise.

3.3.1. Disordered eating and current BMI

The relationship between disordered eating as assessed by the Eating Disorder Inventory (EDI)-3 – Eating Disorder risk scales drive for thinness (DT), bulimia (B) and body dissatisfaction (BD) self report questionnaires (Garner, 2004) and self reported current BMI was tested by multiple regression, with BMI as dependent variable and individual EDI scales as independent (predictor) variables adjusted for age. EDI scales were regressed individually with BMI, as well as together; i.e. with the scores of all three EDI scales as covariates. All associations between EDI

and BMI were regressed with age as a covariate, because each EDI scale was significantly associated with age (scores significantly decrease with age, data presented in the Appendix, page 238), and because age was significantly associated with BMI (BMI increases with age, data presented in the Appendix, Figure 66, page 241). Even though the relationship between the EDI scores and age was not the main objective of this chapter, a short comment was written on the significant association between both in the conclusion and discussion of this chapter (paragraph 3.6.5 *Relationship between EDI scores and age*, page 151), because literature had indicated there were no differences between women of younger and older age groups (Webster and Tiggemann, 2003,Bedford and Johnson, 2006).

3.3.2. Disordered eating and BMI history

I tested whether lowest BMI and/or highest BMI predicted EDI scale scores better than current BMI by multiple regressions, with the individual EDI scales as dependent variables and lowest, current, and highest BMI as independent (predictor) variables adjusted for age. BMIs were regressed individually with the EDI scales, as well as combined in order to test which BMI measure was the best predictor of disordered eating as assessed by the EDI questionnaire.

3.4. *Data description*

A total of 4,050 individuals (3,624 females and 426 males) recruited by the St Thomas' UK Adult Twin Registry took part in this study. They completed the Autumn 2008 questionnaire which can be found online at http://www.twinsuk.ac.uk/phenotypes.html. This questionnaire included 162 questions on several topics including eye surgery, education, work experience, social behaviour, nutritional supplements, eating behaviour, height and weight, physical appearance, lifestyle, diabetes and genetic testing. Of these data 41 questions were available for analyses (see Table 60, page 237 in the Appendix for an overview); 38 of these questions – namely the questions considering EDI and BMI – were analysed in the current chapter. Available questions on breakfast

frequency and waist circumference were excluded since they were beyond the scope of the current chapter. The sample consisted predominantly of females (90%), and for this chapter only females were included (n=3,624). The sample consisted of 780 monozygotic (MZ) twin pairs, 614 dizygotic (DZ) twin pairs and 836 single twins (of who 389 have an MZ and 446 have a DZ twin). The single twins are either single because their twin did not fill out the Autumn 2008 questionnaire or because their twin was male and was excluded from analyses for that reason.

3.4.1. Age

The age ranged from 18-92 with a mean age of 57 years (n=3,624, stdev=12.9, see Figure 22). The distribution of age shows a negative skew; the sample contains relatively few individuals older than 80 years old, at least fewer than expected based on a normal distribution with a mean age of 57; this is skew is probably a life expectancy effect, and individuals older than 80 years are probably less likely to participate in any study.

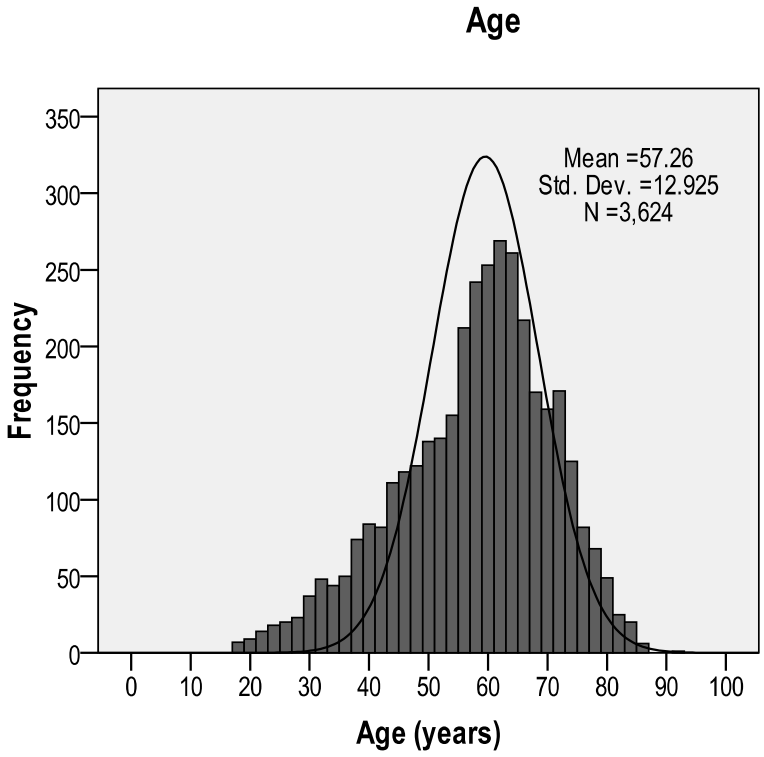

Figure 22: Distribution of age – TwinsUK (females)

3.4.2. Current body mass index (BMI)

Body mass index (BMI) was calculated from self-reported current weight and height (kilogram/meter^2). For some individuals weight was given in both kilograms (kgs) and stones/lbs. Where weight was provided in both units, preference was given to the data in stones/lbs (as this is most commonly used in the United Kingdom (UK) and thus may be more reliable). For eight individuals the standard and metric weight was more than 5kgs apart and they were excluded based on the assumption that one or both of the measurements were erroneous (n=8). An additional four current weight measurements were excluded because they were too low (ranging from 4.5 to 5.8kgs) and thus invalid (n=4). For height again standard and metric measurements were available for some individuals. For five individuals these measurements were more than five centimetres (cms) apart and they were thus excluded (n=5). An additional three measurements were excluded because they were too low (ranging from 0.07 to

0.27 meters, n=3). Distributions of height and weight are presented in the Appendix (see Figure 67 and Figure 68, page 242). In total this resulted in the exclusion of 24 current BMIs, an additional 197 height and/or current weight measurements were missing, rendering a total number of missing BMIs of 221. Valid BMIs range from 7.3 to 73.2 kg/m^2 with a mean of 25.5 (n=3,403, stdev=4.9) (see Figure 23). The distribution of current BMI shows a positive skew; there are more high BMIs than expected based on a normal distribution with a mean of 25.5 (see Figure 23). According to the WHO guidelines a BMI below 18.5 is considered underweight, a BMI between 18.5 and 25 is normal range, between 25 and 30 pre-obese and above 30 obese. In the current sample 85 individuals (2%) would be classified as underweight, 1,780 (52%) as normal range, 1,037 (31%) as pre-obese, and 501 (15%) as obese. Compared to the latest UK Health Survey results in 2007 the current sample contains relatively many women with normal range BMIs and relatively few women classified as obese (see Figure 24, Health Survey for England, 2007).

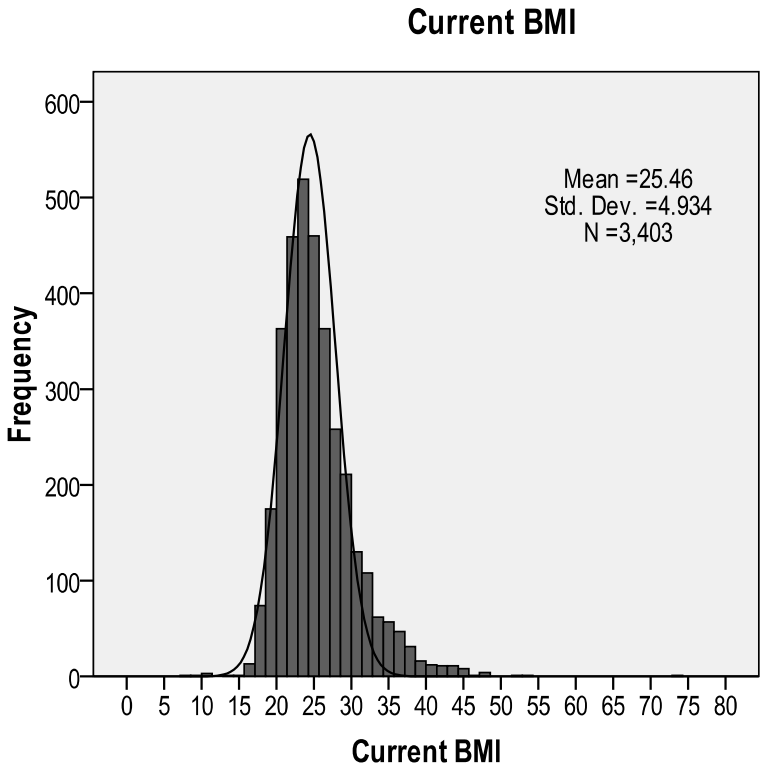

Figure 23: Distribution of current BMI – TwinsUK (females)
There are relatively many high BMIs in the sample based on a normal distribution of the sample mean. The proportion of high BMIs (>35) is however less than the proportion of high BMIs in the UK generally based on the latest UK Health Survey results for women (see Figure 24).

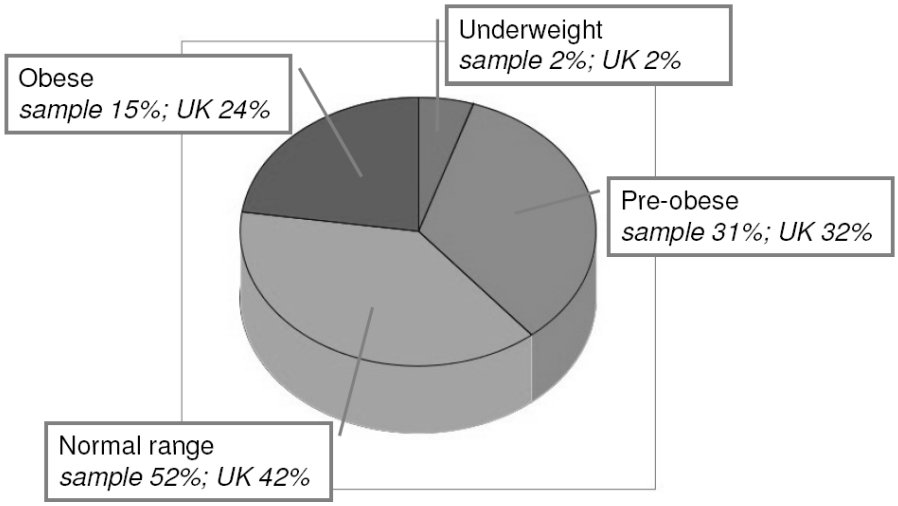

Figure 24: TwinsUK BMI compared to UK BMI (females)
The graph was adapted from the WHO website (http://apps.who.int/bmi). UK data (females) is from the 2007 Health Survey for England (Health Survey for England, 2007).

The sample contains relatively many individuals with normal range BMIs and relatively few obese individuals compared to the UK population.

3.4.3. Lowest adult lifetime BMI

For one individual the standard and metric lowest adult lifetime weight (i.e. lowest weight) measurements were more than 5kgs apart, and this measurement was thus excluded (n=1). An additional two measurements were excluded because they were too low (3.1 and 3.6kgs, n=2), and 14 measurements were excluded because the lowest weight was more than 5kgs higher than the current weight (ranging from 5.4 to 49kgs difference, n=14). A minor difference between lowest adult lifetime and current BMI was allowed accounting for slight differences between standard and metric measurement calculations (maximum difference allowed between lowest and current weight was -4.5kgs or -1.6 BMI units). The distribution of lowest weight is presented in the Appendix (see Figure 69, page 243). In total 198 lowest adult lifetime BMIs (i.e. lowest BMIs) were missing due to invalid and/or missing height and lowest weight measurements. Lowest BMI ranged from 8.5 to 44 with a mean of 20.7 (n=3,426, stdev=3.1) (see Figure 25). For 844 individuals their lowest BMI was within 2 BMI units from their current BMI, 296 individuals were currently more than 10 BMI units heavier than their lowest BMI.

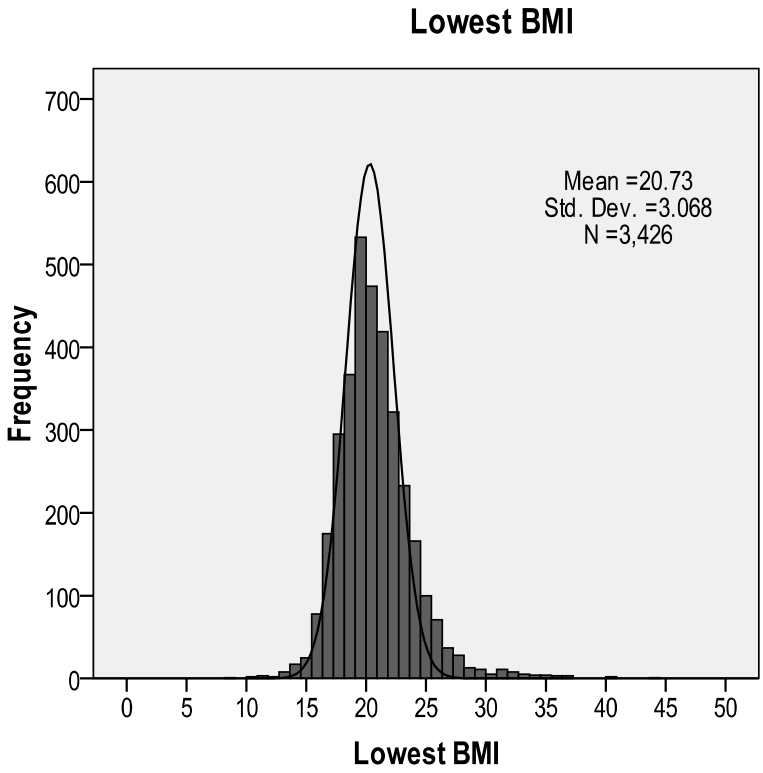

Figure 25: Distribution of lowest adult lifetime BMI – TwinsUK (females)

3.4.4. Highest adult lifetime BMI

For three individuals their given highest weight in kgs was more than 5kgs different from their given highest weight in stones/lbs and their measurements were thus excluded (n=3). A further 35 measurements were excluded because their highest weight was more than 5kgs lower than their current weight. In total 232 highest BMIs were missing because of invalid and/or missing height and highest weight measurements. The distribution of highest weight is presented in the Appendix (see Figure 70, page 243). Highest BMI ranged from 12.8 to 69.7 with a mean of 27.2 (n=3,392, stdev=5.4) (see Figure 26). For most individuals their highest BMI is not more than 2 BMI units higher than their current weight (n=2,177), for 38 individuals their highest BMI is more than 10 BMI units heavier than their current BMI and for 523 individuals their highest BMI is more than 10 BMI units heavier than their lowest BMI. There were 82 individuals who only reported their highest weight and not their current weight, however the total

number of current BMIs reported still outnumbers the number of highest BMIs reported (3,403 vs 3,392).

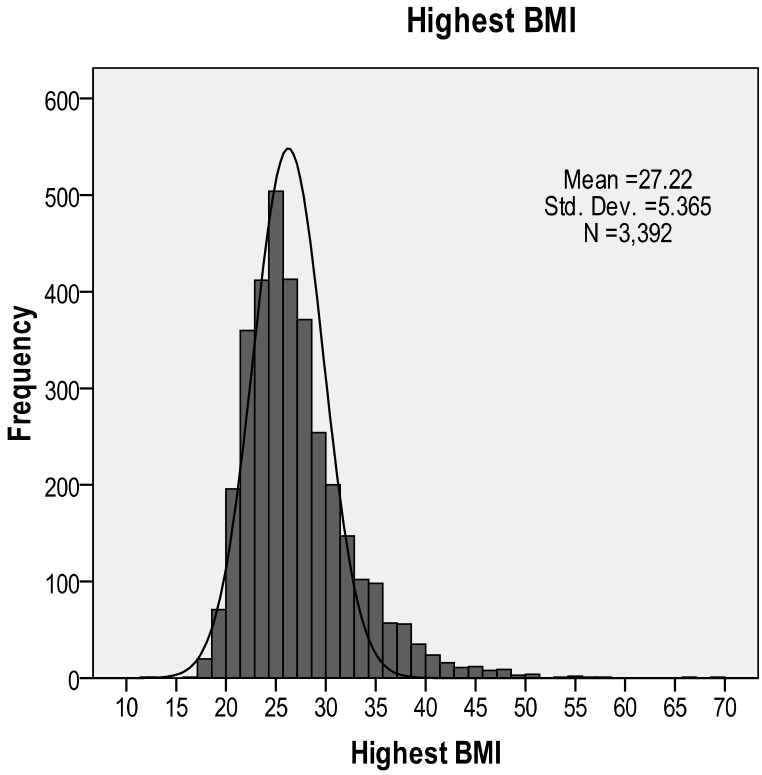

Figure 26: Distribution of highest adult lifetime BMI – TwinsUK (females)

3.4.5. Eating Disorders Inventory

Twenty-five questions from the EDI-3 Eating Disorder Risk scales Drive for Thinness (DT), Bulimia (B), and Body Dissatisfaction (BD) (Garner, 2004) were part of the self report TwinsUK Autumn 2008 questionnaire (see the Appendix for the exact questions, Table 60, page 237). The responses were scored one to six instead of zero to four to ensure a more normal and informative distribution, but in order to be able to compare the scores to the literature the mean following traditional scoring will be presented as well. In addition the mean EDI-1 and EDI-2 scores will be calculated by deleting the question "When I am upset, I worry that I will start eating" from the B scale, and the question "I feel bloated after eating a normal meal" from the BD scale. In Finland the EDI questionnaire was also administered to a twin cohort (Keski-Rahkonen et al., 2005), they

however excluded the question “I like the shape of my buttocks” from the BD scale (personal communication). In order to be able to compare the BD scores to the Finnish study a mean “Finnish-BD” score was calculated as well. If an individual had not completed all questions of the EDI scale they were excluded from analyses, as missing answers would artificially lower the scale score (the proportion of missingness was 4.8%, 3.8% and 6.5% for the DT, B and BD scales respectively). Figure 27 shows the number of individuals skipping one or more EDI questions (see Figure 27). Missingness of answers was not random; it was significantly associated with the mean score of the question with more missing answers for questions with higher mean scores (linear regression: r-square 0.303, p=0.004, n=25 questions, see Figure 28). The questions least and most often skipped, controlling for the mean score of the question, are shown in Table 27. There is no apparent relationship between skipping EDI questions and current BMI (see Figure 29).

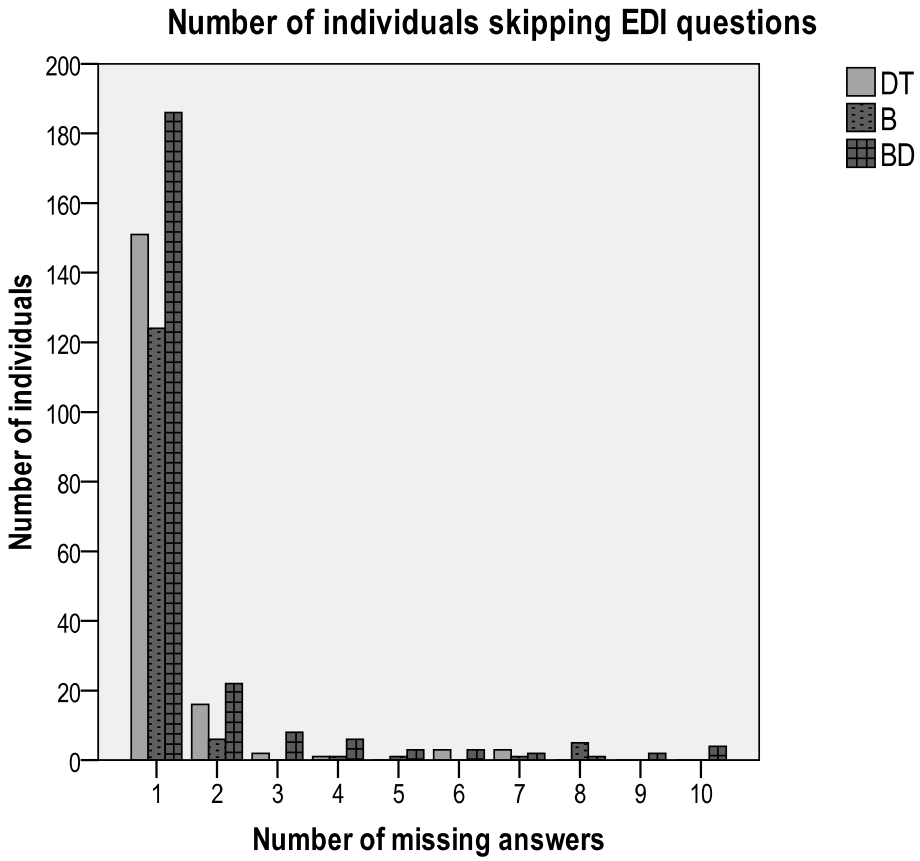

Figure 27: Number of individuals skipping EDI questions
Most individuals skip only one question per EDI scale, very few people skip more than one question. Which question is skipped relates to the mean score of the question (see Figure 28).

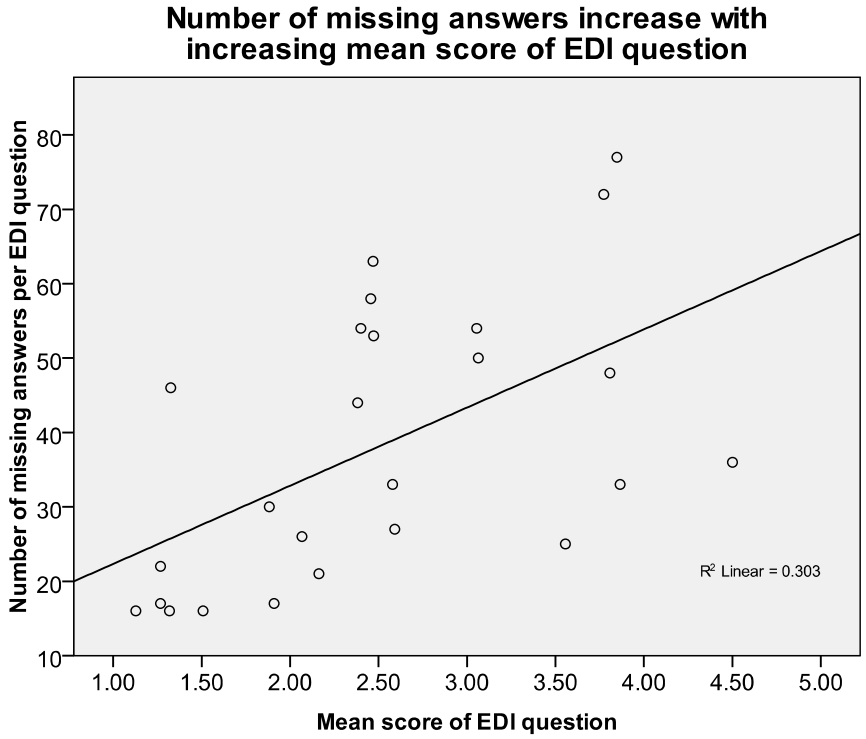

Figure 28: Missingness of answers to EDI questions is not random
The number of missing answers per EDI question significantly increases with increasing mean score of the EDI question (r-square= 0.303, p=0.004, n=25 questions).

Scale	Question	Mean score	N missing	N predicted
Skipped less often than predicted				
BD	I think my stomach is too big	3.56	25	49
BD	I think that my stomach is just the right size	4.50	36	59
BD	I feel satisfied with the shape of my body	3.87	33	52
DTT	If I gain a pound, I worry that I will keep gaining	1.91	17	32
DTT	I am preoccupied with the desire to be thinner	2.16	21	35
Skipped more often than predicted				
BB	I eat or drink in secrecy	1.33	46	26
BB	I eat when I am upset	2.46	58	38
BD	I like the shape of my buttocks	3.77	72	51
BD	I think that my thighs are just the right size	3.85	77	52
DTT	I feel extremely guilty after overeating	2.47	63	38

Table 27: EDI questions skipped least and most often
N predicted is the number of missing answers predicted based on the mean score of the question.

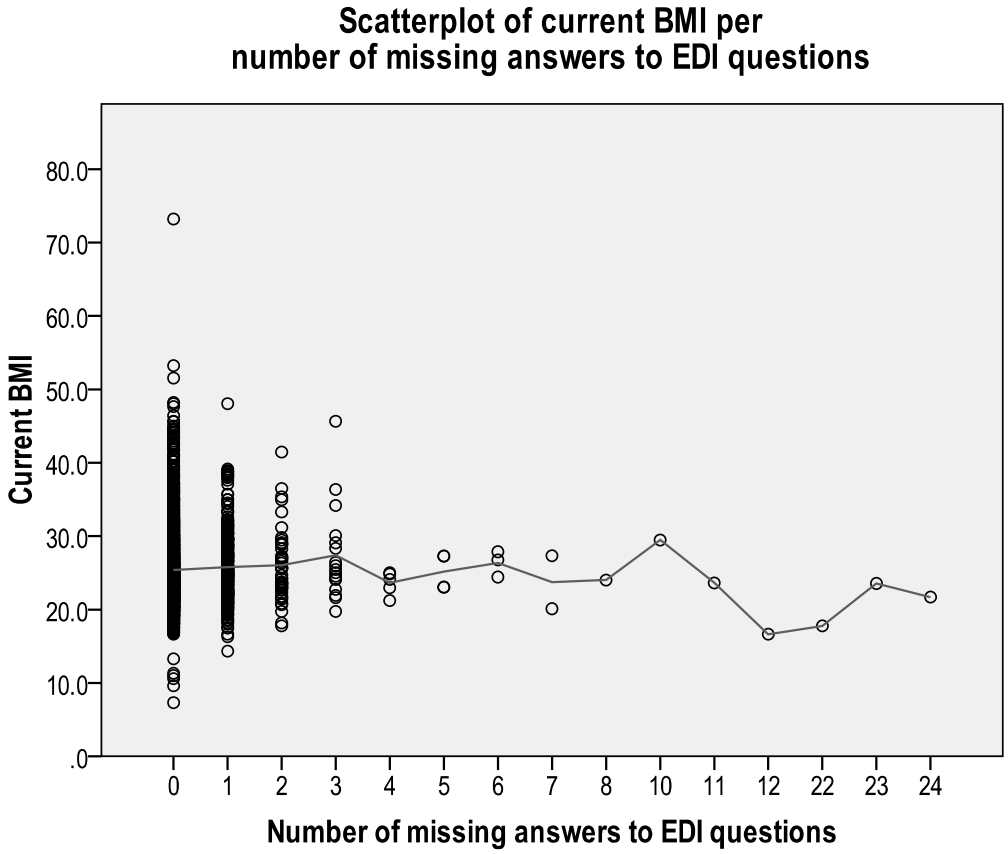

Figure 29: No apparent relationship between current BMI and skipping EDI questions
The interpolation line shows the mean current BMI per number of missing answers. Note: there were three people who skipped all twenty-five EDI questions however for these individuals there were no valid BMI data.

3.4.5.1. Drive for thinness

Drive for thinness (DT) was assessed by seven self-report questions from the EDI-3 Eating Disorder risk scale (Garner, 2004) (see the Appendix Table 60, page 237 for the exact questions). For 178 individuals one or more answers of the DT scale were missing and they were thus excluded from analyses (4.8%). The scores range from 7 to 42, with a mean of 17.7 (n=3,448, stdev=7.1) (see Figure 30). EDI-1 and EDI-2 calculations are equal to the EDI-3 calculation for DT, but following traditional scoring (Garner, 2004) the mean is 5.9 (stdev=5.6).

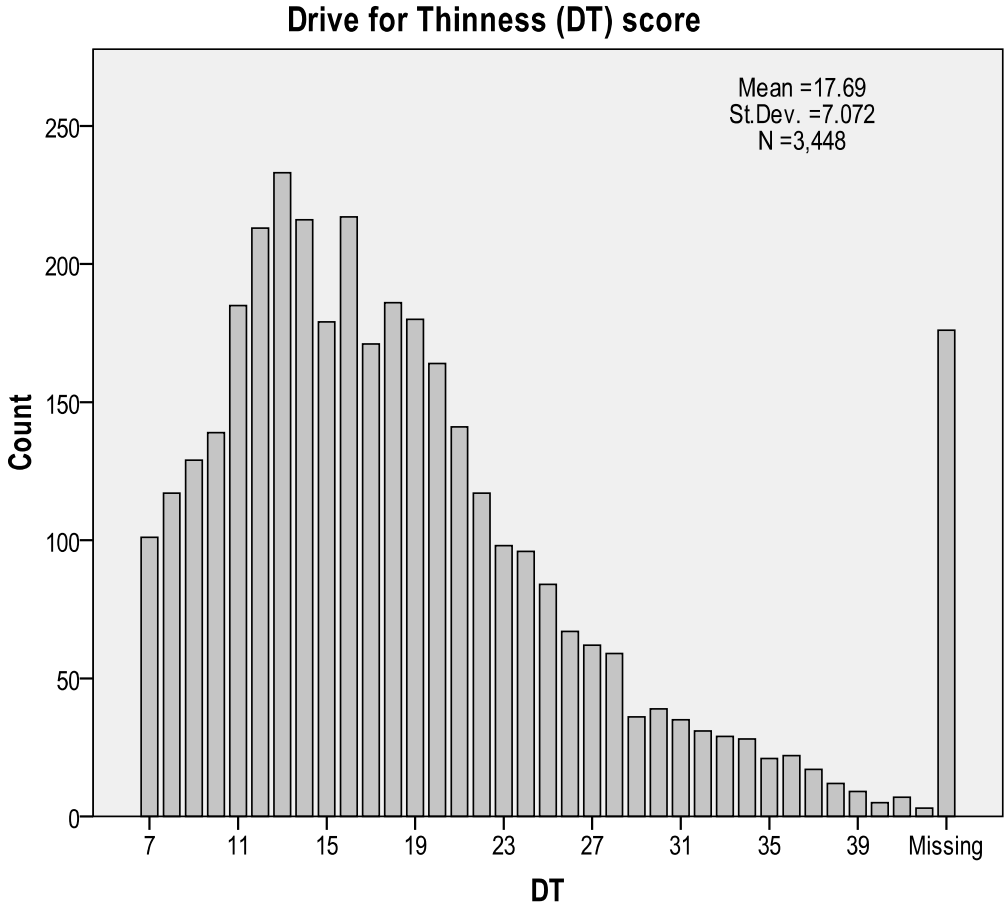

Figure 30: Distribution of DT score – TwinsUK (females)

3.4.5.2. Bulimia

Bulimia (B) was assessed by eight self-report questions from the EDI-3 (see the Appendix Table 60, page 237 for the exact questions). For 138 individuals one or more answers of the B scale were missing and they were thus excluded from analyses (3.8%). The scores range from 8 to 48, with a mean of 12.3 (n=3,486, stdev=4.9) (see Figure 31). With traditional scoring (Garner, 2004) the mean is 1.8 (stdev=3.2), and with EDI-1 and EDI-2 scoring the mean is 10.8 (stdev=4.2). In Chapter 4 this EDI scale was analysed as a dichotomous trait with the 75% percentile, 12, as a threshold.

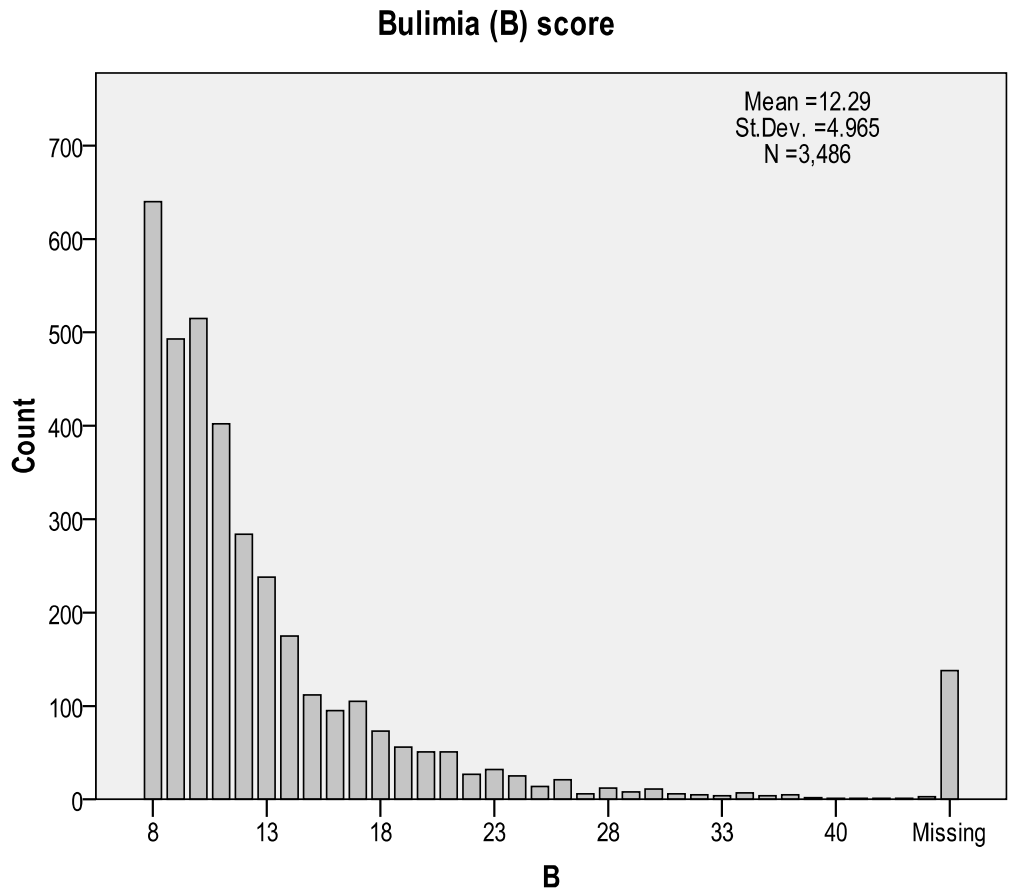

Figure 31: Distribution of B score – TwinsUK (females)

3.4.5.3. Body dissatisfaction

Body Dissatisfaction (BD) was assessed by ten self-report questions from the EDI-3 (see the Appendix Table 60, page 237 for the exact questions). For 237 individuals the answer to one or more questions of the BD scale was missing and they were thus excluded from analyses (6.5%). The scores range from 10 to 60, with a mean of 34.1 (n=3,387, stdev=11.5) (see Figure 32). With traditional scoring (Garner, 2004) the mean is 15.8 (stdev=10.1), with EDI-1 and EDI-2 scoring the mean is 31.6 (stdev=11.2), and the mean Finnish-BD score (see paragraph 3.4.5 *Eating Disorders Inventory*, page 120) is 27.8 (stdev=10).

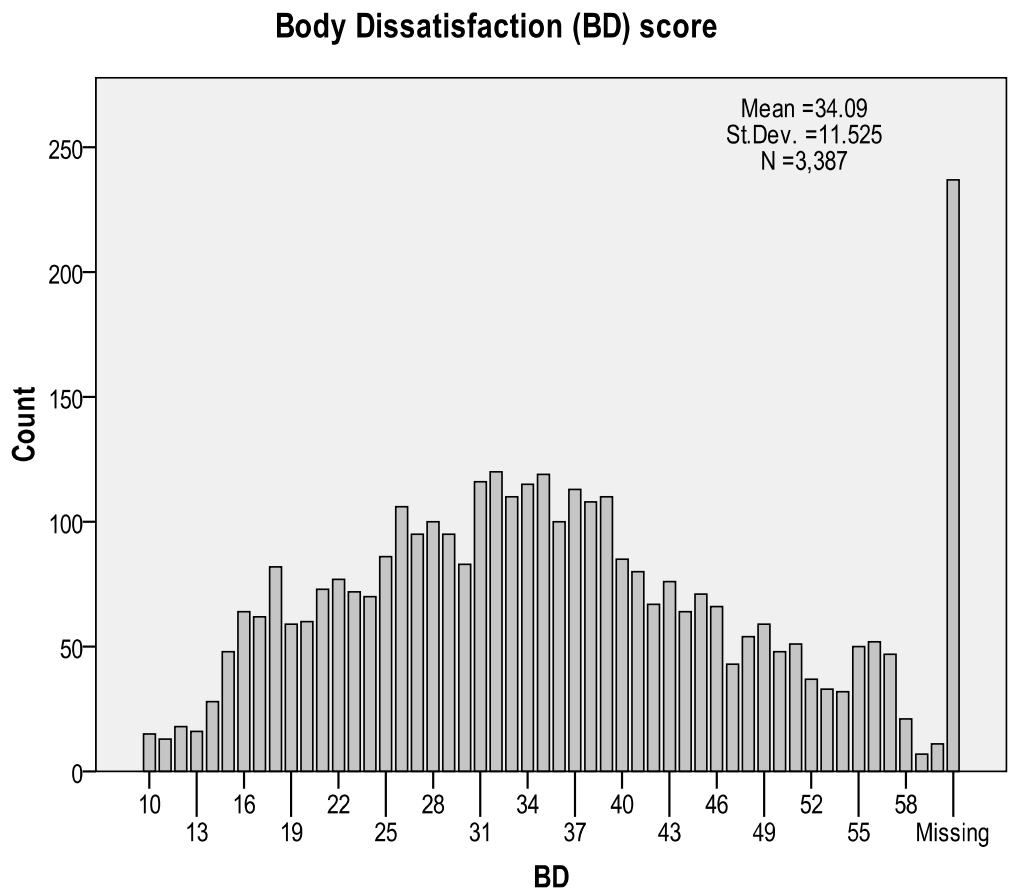

Figure 32: Distribution of BD score – TwinsUK (females)

3.5. *Results*

3.5.1. Disordered eating and current BMI

The EDI Eating Disorder Risk scales Drive for Thiness (DT), bulimia (B), and body dissatisfaction (BD) scores are all significantly associated with current BMI (controlling for age, $p<0.001$), with higher scores predicting higher current BMIs (see Table 28 and Figure 33 to Figure 35). BD explains most variation in current BMI; it has the highest standardized coefficient (beta) (0.53, see Table 28). This means that when predicting current BMI, one standard deviation increase in BD score increases the current BMI with 0.53 standard deviations.

Results predicting current BMI by EDI-3

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
DT	(Intercept)	18.32	0.49			
	Age	0.05	0.01	0.13	53.49	2.60E-13
	DT	0.24	0.01	**0.34**	286.51	0.00E+00
B	(Intercept)	17.99	0.56			
	Age	0.06	0.01	0.16	71.00	3.58E-17
	B	0.32	0.02	**0.32**	177.45	0.00E+00
BD	(Intercept)	15.08	0.43			
	Age	0.05	0.01	0.12	53.65	2.39E-13
	BD	*0.23*	*0.01*	***0.53***	*911.93*	*0.00E+00*

Table 28: Results predicting current BMI by EDI-3 scales adjusted for age
The results are grouped per EDI scale; DT, B, and BD. The variables column shows that all analyses were adjusted for age. The third column, labelleled 'B', indicates the correlation coefficient between the EDI scale and current BMI; SE B is the standard error of this coefficient. Beta is the standardised coefficient (the coefficient divided by the standard deviaton); the strength of association of the different EDI scales be compared to each other based on the beta values, because the beta does not depend on the unit of measurement. The Wald Chi-square is also known as the Wald statistic, or simply 'chi-square'. The p value is the p value adjusted for age (the p value of age is adjusted for the EDI scale, and it can be seen that age is significantly associated to current BMI). Note: the p value 0.00E+00 indicates that the p value was lower than the lowest p value that could be displayed by SPSS ($p < 1.00E-15$), i.e. these p values were highly significant. All three EDI-3 scales are significantly associated with current BMI, but BD explains most variation in BMI as indicated by the highest beta value (0.53, indicated in *italic*).

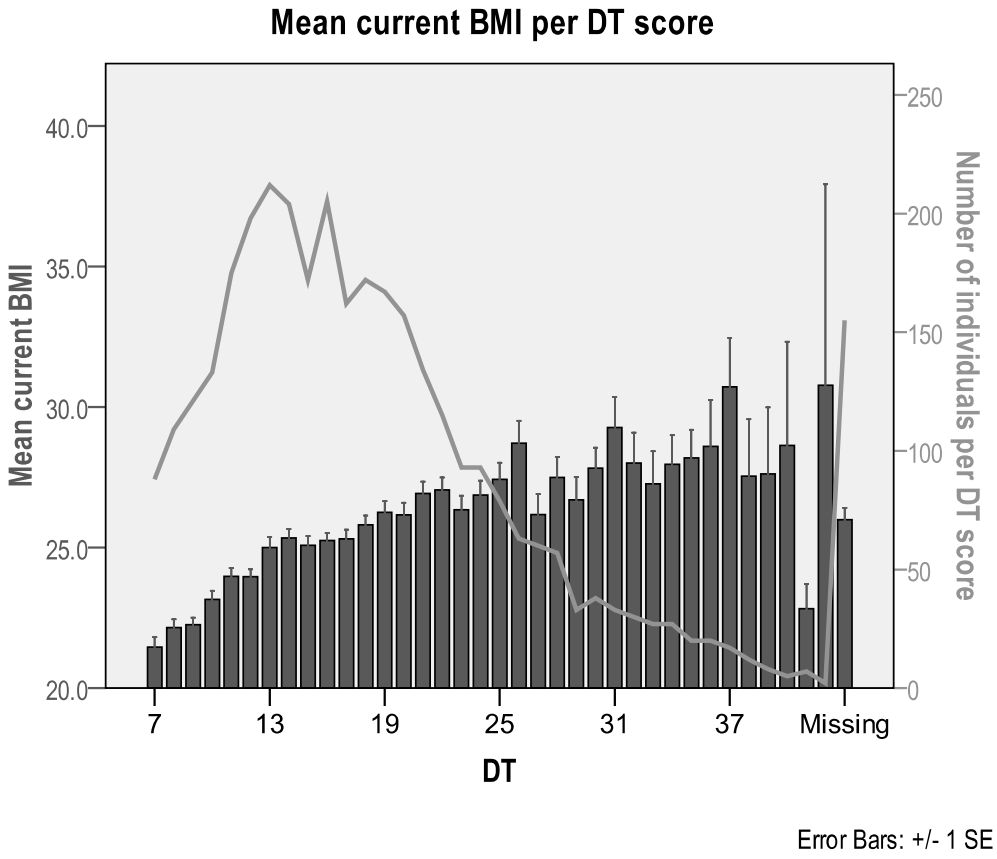

Figure 33: Current BMI increases with increasing DT scores
The left Y axis and the bars indicate the mean current BMI (± one standard error of the mean (SE)) per DT score. The right Y axis and the line show the distribution of DT scores, i.e. the number of individuals per DT score.

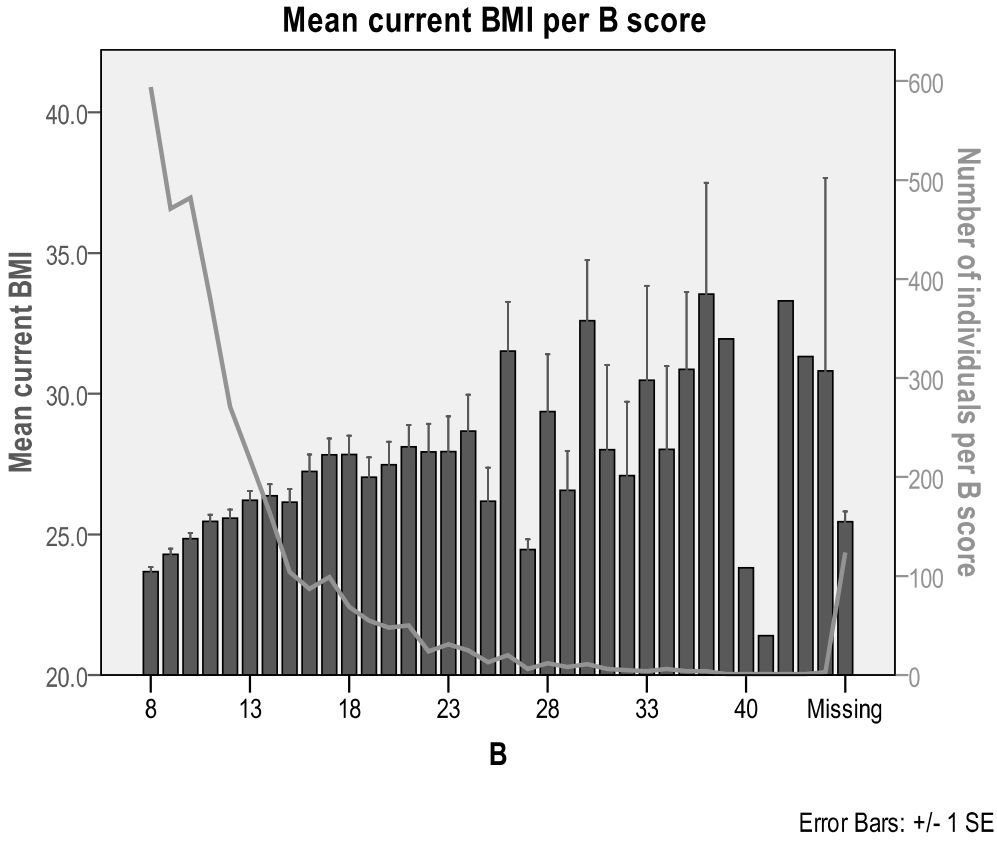

Figure 34: Current BMI increases with increasing B scores
The left Y axis and the bars indicate the mean current BMI (± one standard error of the mean (SE)) per B score. The right Y axis and the line show the distribution of B scores, i.e. the number of individuals per B score. There are very few individuals with high bulimia (B) scores.

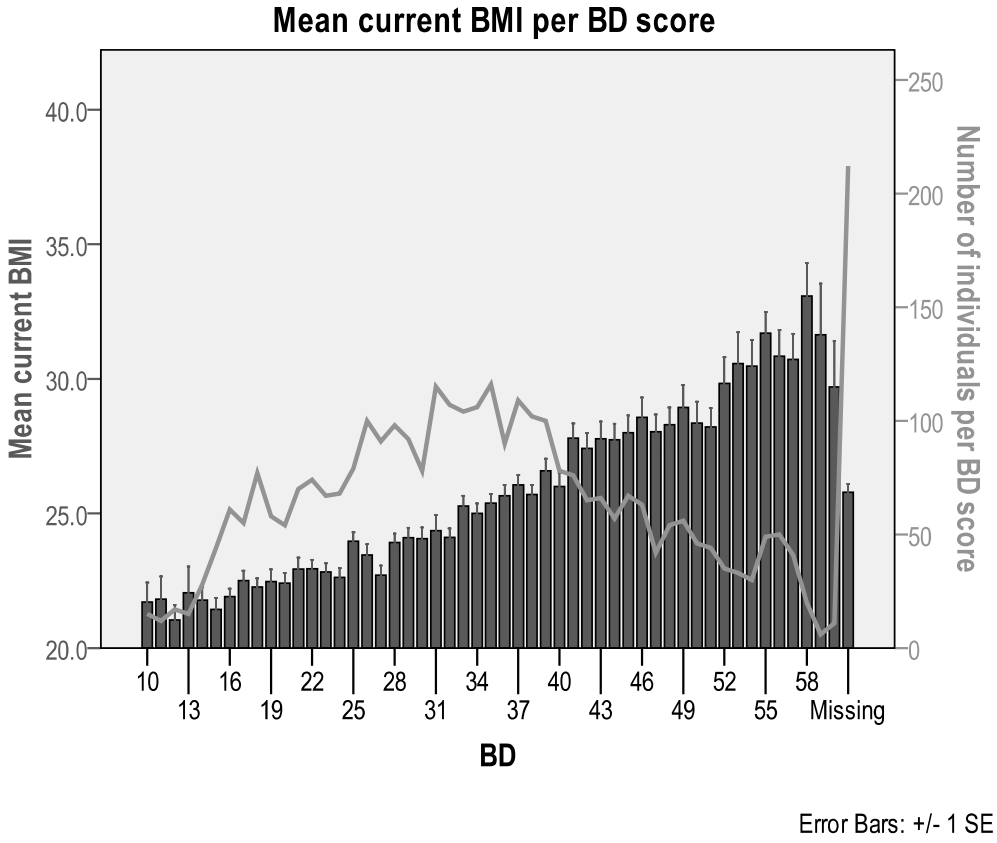

Figure 35: Current BMI increases with increasing BD scores
The left Y axis and the bars indicate the mean current BMI (± on standard error of the mean (SE)) per BD score. The right Y axis and the line show the distribution of BD scores, i.e. the number of individuals per BD score.

The different EDI scales are however moderately correlated (see Table 29), and from the combined multiple regressions it becomes apparent that they partly explain the same variation in current BMI. When the three EDI scales are regressed together to predict current BMI BD explains most unique variation in current BMI (beta=0.51, p<0.001), B also significantly contributes to the model (beta=0.10, p=1.84E-04), but DT does not (p=0.206) (see Table 30).

Correlations

	DT	B	BD
DT	1	0.595	**0.623**
B	0.595	1	0.472
BD	**0.623**	0.472	1

Table 29: Correlations of the EDI-3 scales
Partial correlations adjusted for age. The strongest correlation is between DT and BD (0.623, in **bold**), but there is also considerable correlation between DT and B, and B and BD.

Results predicting current BMI by EDI-3 scales combined

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
All combined	(Intercept)	14.14	0.51			
	Age	0.05	0.01	**0.14**	65.63	5.43E-16
	DT	-0.02	0.02	**-0.03**	1.60	2.06E-01
	B	*0.10*	*0.03*	***0.10***	*13.99*	*1.84E-04*
	BD	*0.22*	*0.01*	***0.51***	*503.24*	*0.00E+00*

Table 30: Results predicting current BMI by all EDI-3 scales combined
Beta (in **bold**) is the standardised correlation coefficient; i.e. the coefficient based on which the variables can be compared, as beta does not depend on the unit of measurement. The unstandardised coefficient (B) and standard error of the unstandardised coefficient (SE B) are shown as well. When all EDI scales are controlled for BD explains most unique variation in current BMI (beta=0.51, indicated in *italic*), B also has a significant contribution (beta=0.10, in *italic*), but DT does not significantly contribute to the model. Note: the p value 0.00E+00 indicates the p value was lower than the lowest p value that could be displayed in SPSS, i.e. this association is highly significant.

3.5.1.1. Assessing the prediction of current BMI

Current BMI can be predicted significantly better using the EDI-3 Risk scale scores than when using the current BMI mean as a 'best guess', however the difference between the predicted and observed current BMI still has a large range (see Table 31). BD is the best predictor of current BMI, and adding B and DT to the model only improves the model marginally. The standard deviation of the raw residuals is however smallest for the model including all three EDI-3 scales (see Table 31), and this model will thus be considered the best model to predict current BMI. The residuals are normally distributed (see Figure 36), and characteristics of the outliers (± two standard deviations of the mean) are presented in Table 32. Only 3.8% of the cases have a residual value ± two

standard deviations, and 1.2% of the cases have a residual value ± three standard deviations, which is within 0.5% of what would be expected for an ordinary sample indicating that the assumption of normally distributed errors for regression models has not been violated. The negative outliers (i.e. individuals for whom the observed current BMI was much lower than the predicted) have a lower lowest and current BMI (17.0 and 16.6 versus 20.6 and 25.0 respectively, see Table 32); the positive outliers have higher lowest, current, and highest BMI (see Table 32). The model thus performs well predicting current BMIs in the normal range, but performs less well to predict very low and very high current BMIs. Figure 37 shows that current BMI was more often underestimated than overestimated, which is a result of the fact that the sample contained more high BMIs than low BMIs (see distribution of current BMI Figure 23, page 117). The variance in residuals slightly increases for increasing BD scores indicating that the prediction of current BMI gets slightly less accurate with higher BD scores (see Figure 37). For 60% of the individuals the prediction of current BMI is ± 8.5 BMI units of the observed current BMI (see Figure 37).

Raw residuals per model predicting current BMI

Model	*N*	*Minimum*	*Maximum*	*Stdev*
DT	3248	-20.42	49.30	4.61
B	3279	-19.47	48.66	4.71
BD	*3191*	*-23.45*	*48.82*	*4.20*
BD and B	*3093*	*-23.44*	*48.94*	*4.21*
BD, B and DT	***2981***	***-23.40***	***48.88***	***4.19***

Table 31: Raw residuals per model predicting BMI
All models were adjusted for age. BD is the best predictor of current BMI, adding B and DT to the model only improves the model marginally. The standard deviation (Stdev) of raw residuals is smallest for the model including all three EDI-3 scales, and this will thus be considered the best model to predict current BMI (stdev=4.19, in ***bold***). The raw residuals (observed current BMI minus predicted) range from -23.40 to +48.88.

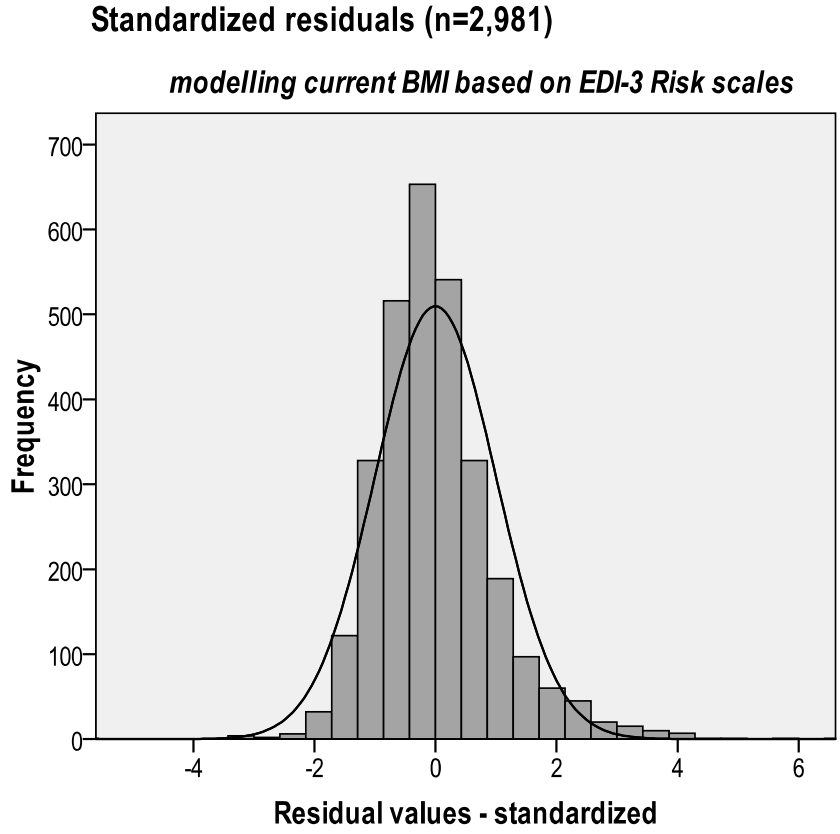

Figure 36: Distribution of standardised residuals for the model predicting current BMI
The residuals are normally distributed and no more than 5% of the cases have a residual value more than 2 standard deviations away from the sample mean.

Characteristics outliers modelling current BMI

	General sample (n=3487)	*Stdev*	Outliers - 2 stdev (n=19)	*Stdev*	Outliers + 2 stdev (n=118)	*Stdev*
Age	57	13.0	52	12.0	55	12.0
DT	*18*	*7.0*	*25*	*10.0*	*21*	*7.0*
B	*12*	*5.0*	*19*	*10.0*	*15*	*7.0*
BD	*34*	*11.0*	*44*	*12.0*	*41*	*11.0*
Lowest BMI	***20.6***	*3.0*	***17.0***	*2.0*	***26.4***	*5.0*
Current BMI	***25.0***	*4.0*	***16.6***	*5.0*	***39.2***	*5.0*
Highest BMI	*26.8*	*5.0*	*22.4*	*3.0*	***41.5***	*6.0*

Table 32: Characteristics of outliers for the model predicting current BMI
The outliers are of approximately equal age, and have slightly elevated EDI-3 scores compared to rest of the sample (in *italic*). Negative outliers (-2 stdev, n=19) have lower mean lowest and current BMIs (middle column, in ***bold***) and the positive outliers (+2 stdev, n=118) have higher lowest, current, and highest BMI (most right column, in ***bold***).

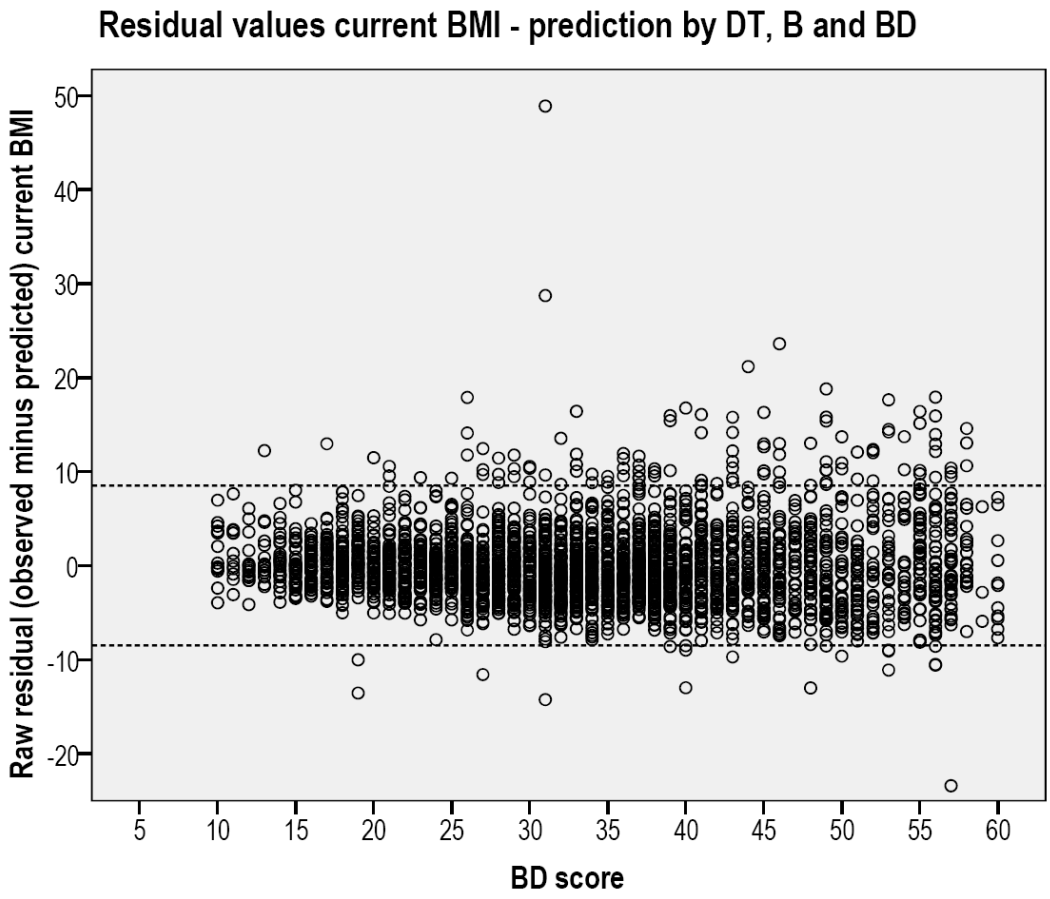

Figure 37: Residual values of the model predicting current BMI per BD score
Raw residuals were plotted with BD score because it was the most important predictor of the model. The dotted lines indicate a range of two standard deviations from the mean. There are more positive residuals than negative residuals, i.e. the current BMI was more often underestimated than overestimated. The characteristics of the outliers are given in Table 32. The variance in residual values appears to increase slightly for higher BD scores, indicating that the prediction of current BMI is less accurate for higher BD scores.

3.5.2. Disordered eating and BMI history

I tested whether lowest and/or highest BMI would predict disordered eating as assessed by the EDI-3 scales better than current BMI. Lowest, current and highest BMI are however correlated, with current and highest BMI being highly correlated (0.909, see Table 33). Indeed when predicting DT based on all BMIs only highest BMI explains a significant proportion of unique variation (see Table 34), combining highest BMI with only lowest or only current BMI gives the same result (data not shown) thus the best model to predict DT scores is based on highest BMI only adjusted for age (beta=0.38, see Table 34). Figure 38 shows the

relationship between lowest, current, and highest BMI with DT scores. The results for predicting B follow the same pattern, only highest BMI explains a significant proportion of unique variation in B scores (see Table 35 and Figure 39).

Correlations

	Lowest BMI	Current BMI	Highest BMI
Lowest BMI	1	0.620	0.624
Current BMI	0.624	1	**0.909**
Highest BMI	0.620	**0.909**	1

Table 33: Correlations for lowest, current, and highest BMI
Partial correlations adjusted for age. Current and highest BMI are very strongly correlated (0.909, indicated in **bold**). Lowest and current BMI, and lowest and highest BMI are moderately correlated.

Results predicting DT by age, lowest, current and highest BMI combined

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
DT	(Intercept)	10.15	1.08			
	Age	-0.10	0.01	-0.18	96.09	0.00E+00
	Lowest BMI	0.03	0.06	0.01	0.22	6.40E-01
	Current BMI	0.04	0.08	0.03	0.28	5.97E-01
	Highest BMI	*0.47*	*0.07*	*0.36*	*43.00*	*5.48E-11*

Results predicting DT by highest BMI

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
DT	(Intercept)	9.98	0.91			
	Age	-0.10	0.01	-0.18	97.49	0.00E+00
	Highest BMI	*0.49*	*0.02*	***0.38***	*395.49*	*0.00E+00*

Table 34: Results prediction DT by BMI history
All analyses were adjusted for age. The columns B and SE B represent the unstandardised coefficients, and standard errors of the unstandardised coefficients, of association. Beta is the standardised coefficient. Lowest, current, and highest BMI are correlated and partly explain the same variation in DT scores. Only highest BMI explains a significant proportion of unique variance in DT scores (upper model, in *italic*), its beta of 0.38 (lower model, in ***bold***) indicates that when predicting DT one standard deviation increase in highest BMI increases DT by 0.38 standard deviations.

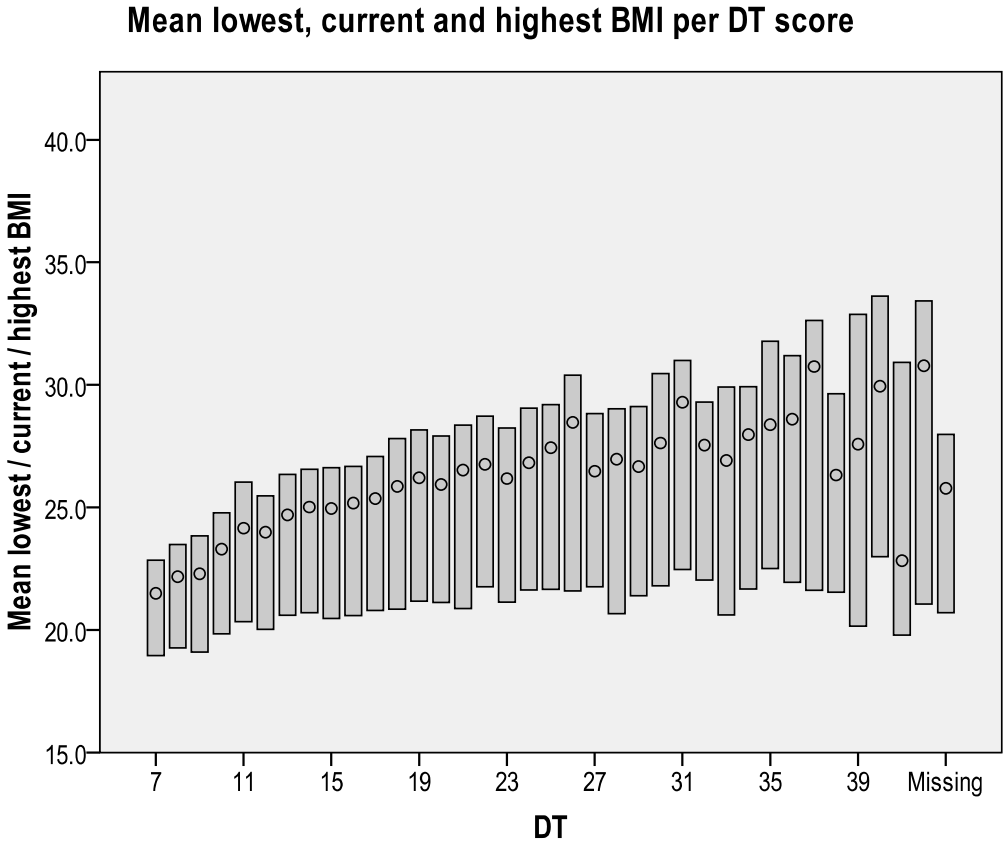

Figure 38: Mean lowest, current, and highest BMI per DT score
The circles indicate the mean current BMI per DT score; the upper line of the bar indicates the mean highest BMI and the lower line of the bar indicates the mean lowest BMI per DT score. With increasing DT scores the means of the BMIs increase as well, which is most prominent in highest BMI. Missingness is not related to BMI (see most right bar, and 3.4.5 *Eating Disorders Inventory*, Figure 29, page 124).

Results predicting B by age, lowest, current and highest BMI combined

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
B	(Intercept)	10.08	0.81			
	Age	-0.11	0.01	-0.28	202.33	0.00E+00
	Lowest BMI	-0.01	0.04	-0.01	0.06	8.03E-01
	Current BMI	0.00	0.68	0.00	0.00	9.84E-01
	Highest BMI	*0.32*	*0.06*	*0.34*	*24.73*	*6.60E-07*

Results predicting B by highest BMI

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
B	(Intercept)	9.92	0.67			
	Age	-0.11	0.01	-0.28	204.66	0.00E+00
	Highest BMI	*0.31*	*0.02*	***0.34***	*214.99*	*0.00E+00*

Table 35: Results prediction B by BMI history
All analyses were adjusted for age. Lowest, current, and highest BMI partly explain the

same variation in B scores. Only highest BMI explains a significant proportion of unique variance in B scores (upper model, in *italic*), its beta of 0.34 (lower model, in ***bold***) indicates that when predicting B one standard deviation increase in highest BMI increases B by 0.34 standard deviations.

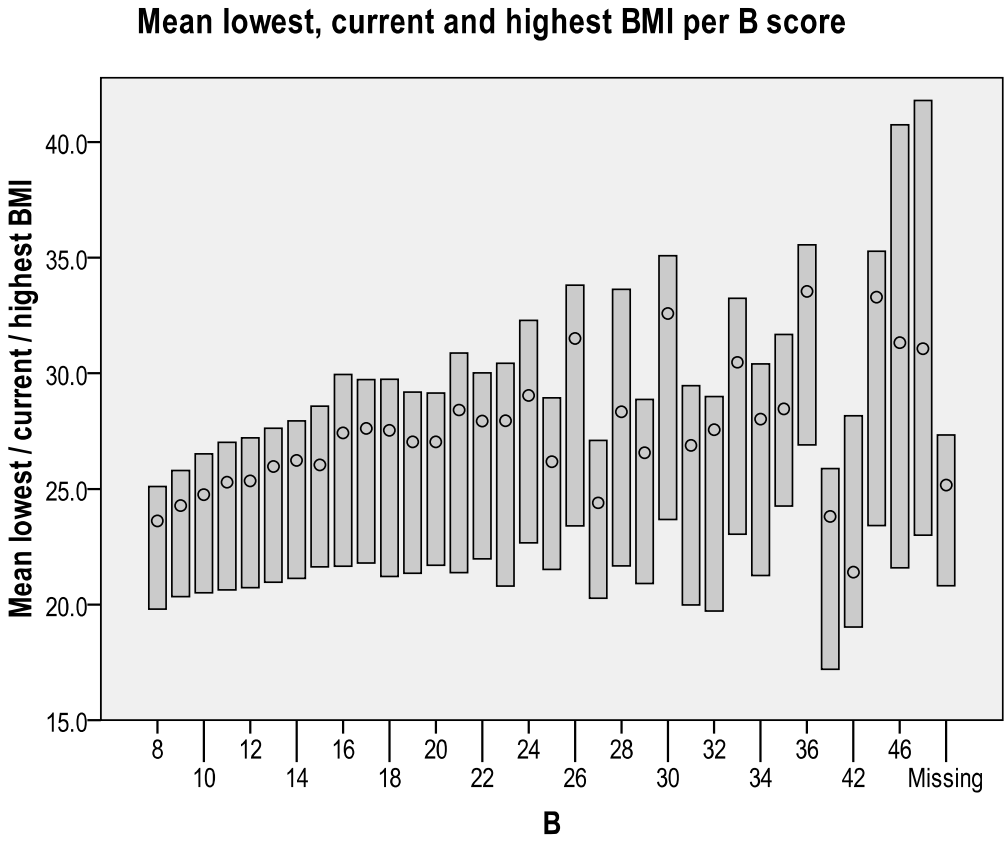

Figure 39: Mean lowest, current, and highest BMI per B score
The circles indicate the mean current BMI per B score; the upper line of the bar indicates the mean highest BMI and the lower line of the bar indicates the mean lowest BMI per B score. The BMIs increase with increasing B scores, however among the higher B scores there is more variation in BMI, which can be explained by the fact that there were very few individuals with high B scores in the sample (see Figure 31, page 126). Missingness is not related to BMI (see most right bar, and Figure 29, page 124).

For BD lowest, current, and highest BMI all significantly contribute to the prediction of BD scores ($p<0.005$, see Table 36 and Figure 40). In contrast to the results for DT and B current BMI, not highest BMI, explains most variance in BD scores. Based on this model an individual's BD score can be predicted by multiplying their age, lowest -, current -, and highest BMI by the corresponding unstandardised coefficient (B, Table 36) and adding them to the unstandardised

coefficient of the intercept (B=8.97, see Table 36). Table 37 provides an example of this calculation.

Results predicting BD by age, lowest, current and highest BMI

EDI scale	*Variables*	*B*	*SE B*	***Beta***	*Wald Chi-square*	*p*
BD	(Intercept)	8.97	1.69			
	Age	-0.11	0.02	-0.12	49.10	2.43E-12
	Lowest BMI	*-0.27*	*0.09*	***-0.07***	*9.87*	*1.68E-03*
	Current BMI	*1.18*	*0.10*	***0.50***	*144.28*	*0.00E+00*
	Highest BMI	*0.25*	*0.09*	***0.12***	*7.89*	*4.98E-03*

Table 36: Results prediction BD by BMI history
All analyses were adjusted for age. Beta is the standardised coefficient. The BMIs explain more variation in BD than in the other EDI-3 scales; i.e. the beta values are higher for BD than for DT or B (see Table 34 and Table 35). Current BMI explains most variation in BD scores (beta=0.50), but both lowest and highest BMI significantly contribute to the model (p<0.005, in *italic*). Based on this model an individual's BD score can be predicted by multiplying their age, lowest -, current -, and highest BMI by the unstandardised coefficients value (B) and adding them to the intercept B value (see Table 37 for an example).

Example calculation: prediction BD score

Variables	*B*	*Individual data (participant #83212)*	*B * individual data*
(Intercept)	8.97		8.97
Age	-0.11	64	-6.78
Lowest BMI	-0.27	21.3	-5.81
Current BMI	1.18	28.7	33.72
Highest BMI	0.25	30.5	7.72
		Predicted BD	*38*
		Observed BD	*39*
		Raw residual	*1.19*

Table 37: Example calculation of predicted BD score by BMI history
B is the unstandardised correlation coefficient. The BD score can be predicted by multiplying an individual's variable values by the corresponding unstandardised coefficient (B) of the model. Participant #83212 was chosen randomly, for this person the predicted BD score was calculated by multiplying the her scores by the unstandardised coefficients (B) of the model and adding them to the intercept; the predicted BD score was only 1.19 BD units lower than the observed BD score (raw residual=1.19, in *italic*).

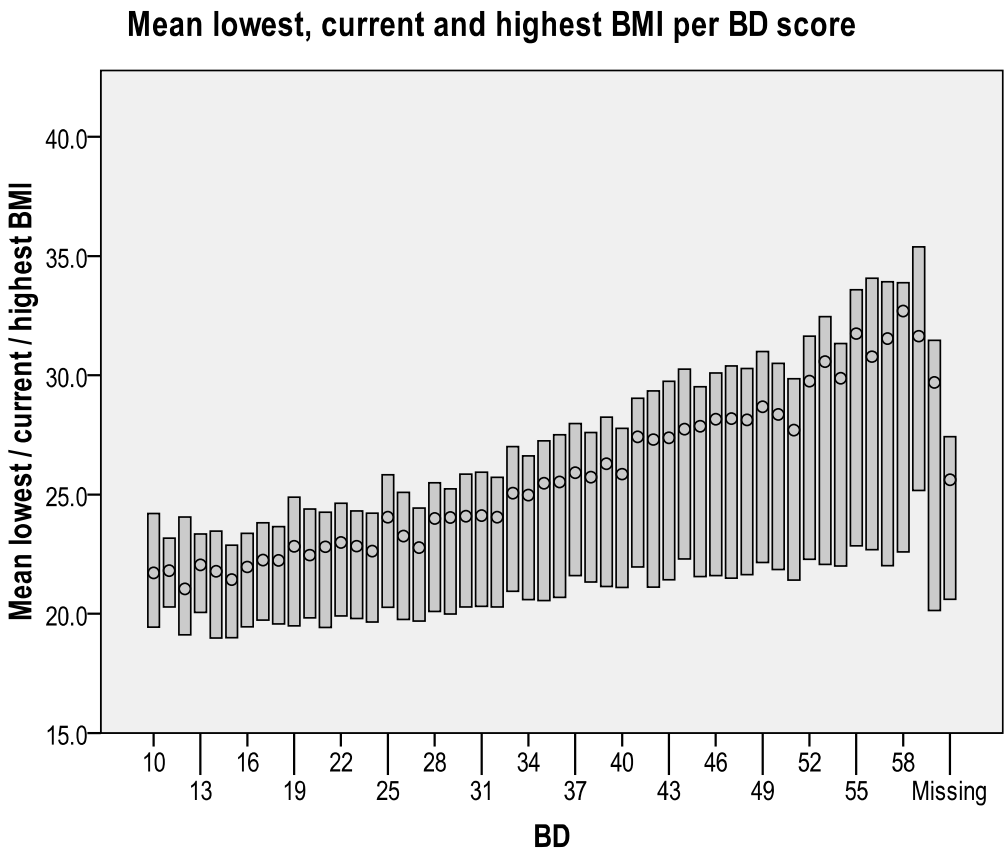

Figure 40: Mean lowest, current, and highest BMI per BD score
The circles indicate the mean current BMI per BD score; the upper line of the bar indicates the mean highest BMI and the lower line of the bar indicates the mean lowest BMI per BD score. The BMIs increase with increasing BD scores, most prominently for current and highest BMI. Missingness is not related to BMI (see most right bar, and Figure 29, page 124).

3.5.2.1. Assessing the prediction of disordered eating

In general BD is most accurately predicted out of the EDI-3 scales (see Table 38). The EDI-3 scores can be predicted significantly better based on their BMI than when using the scale mean as a 'best guess', however the prediction is not equally accurate for all individuals; there are subgroups for whom the prediction is not as good as it is for the majority of the sample. For DT this is apparent from the fact that the standardised residuals are not normally distributed; there are more cases with negative residual values than predicted based on a normal distribution and there are relatively many outliers with high positive residuals

(see Figure 41). The number of individuals with residual values ± two and three standard deviations from the sample mean is however not larger than 5% and 1% respectively, which are the expected percentages for a normally distributed sample. The characteristics of the outliers ± two standard deviations from the sample mean are given in Table 39; negative residual outliers have a higher mean current and highest BMI (n=10) and positive residual outliers stand out for having elevated EDI-3 scores (n=159, see Table 39). Figure 42 shows the subgroup of individuals with high positive residual values (dotted circle, see Figure 42); they have a normal range highest BMI and high positive residual values indicating that their observed DT score was much higher than their predicted score. The mean DT score significantly increases with increasing highest BMI, and on average people with normal range highest BMIs have low DT scores, but this does not hold true for this subgroup of individuals. Omitting this subgroup from the sample would normalise the distribution of standardised residuals and increase the predictive ability of the model for the rest of the sample.

Raw residuals per model predicting EDI-3

EDI scale	*Prediction by*	*N*	*Minimum*	*Maximum*	*Stdev*
DT	Highest BMI	3243	-16.34	25.12	6.45
B	Highest BMI	3117	-65.95	28.36	9.74
BD	Lowest, current, and highest BMI	3129	-12.40	33.08	4.46

Table 38: Raw residuals of the models best predicting the EDI-3 scale scores
The best model to predict DT and B scores is based on highest BMI adjusted for age, in contrast to BD for which the model based on lowest, current, and highest BMI is the better model. The range of raw residuals (i.e. observed minus predicted score) is however considerable. BD can be predicted most accurately; it has the lowest standard deviation (Stdev, 4.46, bottom row).

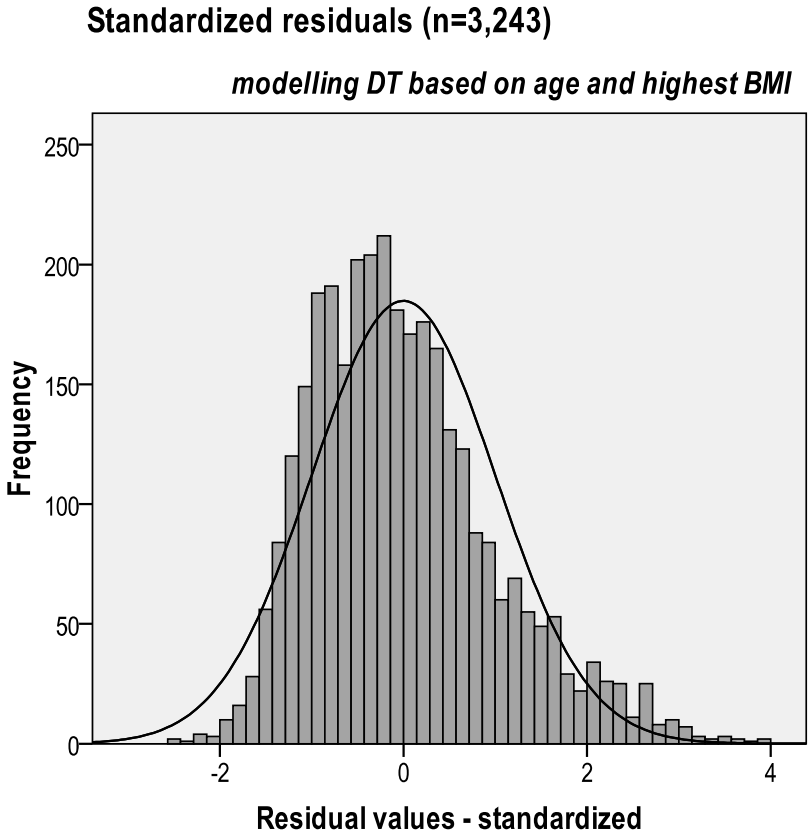

Figure 41: Distribution of standardised residuals – DT model based on BMI history
The distribution of standardised residuals deviates from normality; there are more negative residuals than expected based on a normal distribution and there are outliers with high positive residual values.

Characteristics outliers modelling DT

	General sample (n=3455)	*Stdev*	Outliers - 2 stdev (n=10)	*Stdev*	Outliers + 2 stdev (n=159)	*Stdev*
Age	57	12.9	58	13.1	54	12.6
Lowest BMI	21	3.1	23	3.7	20	2.8
Current BMI	27	5.4	***40***	*6.3*	28	4.5
Highest BMI	25	4.9	***32***	*5.6*	26	4.4
DT	16.9	6.1	*9.4*	*2.5*	***34.5***	*3.1*
B	12.0	4.6	*9.8*	*2.2*	*19.1*	*7.9*
BD	33.5	11.3	32.8	15.2	***46.2***	*9.5*

Table 39: Characteristics outliers - model DT based on BMI history
The outliers are of approximately equal age. Negative outliers (-2 stdev, n=10) have higher mean current and highest BMIs (middle column, in ***bold***), and the positive outliers (+2 stdev, n=159) have elevated EDI-3 scores (most right column, in ***bold***).

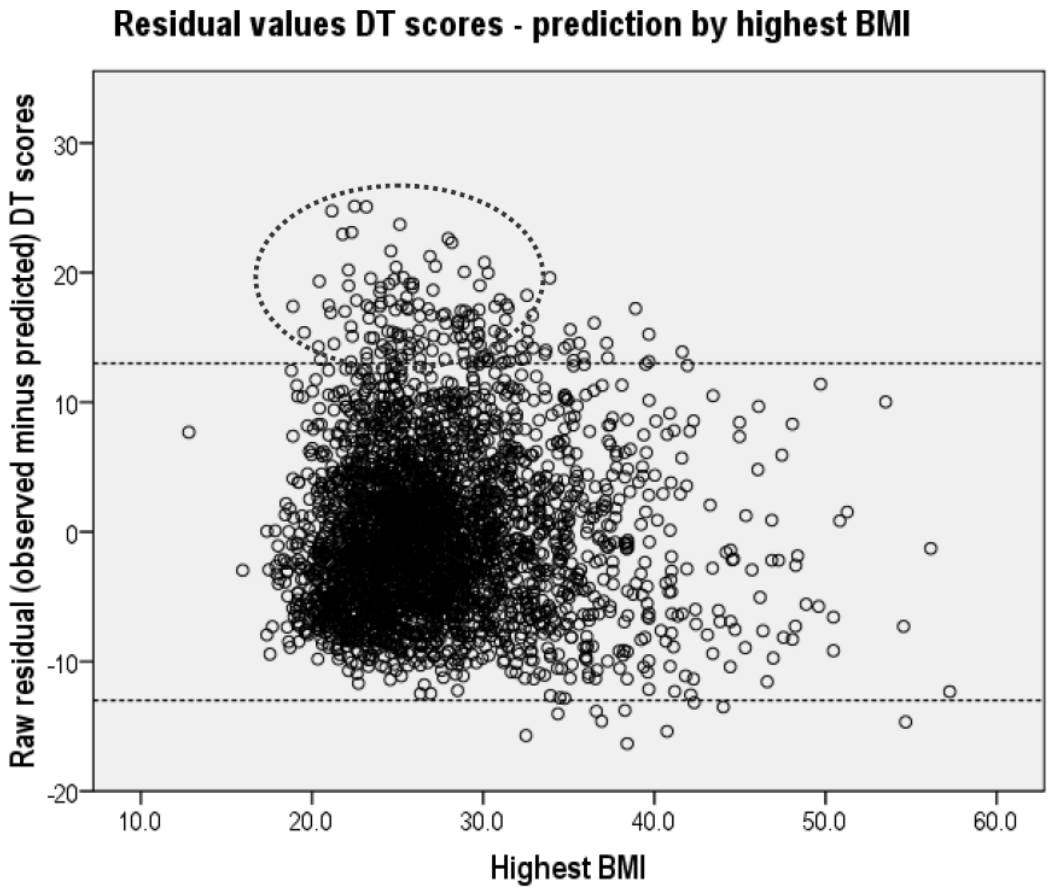

Figure 42: Raw residuals DT model based on BMI history
There is a subgroup of individuals for whom the DT score is underestimated (indicated by the dotted circle); based on their highest BMI their DT score was predicted to be lower than it actually was. Dotted lines indicate two standard deviations; for 60% of the sample the predicted DT score was ± 13 units of the observed score.

The standardised residuals of the B scores are normally distributed (see Figure 43). Characteristics of the cases most different from the sample mean, i.e. individuals for whom the model predicted the B score least accurately, are given in Table 40; it concerns individuals with lower and higher than average EDI-3 scores. Residuals are equally distributed on each increment of the predictor variable; however the prediction is fairly inaccurate with the predicted B score deviating from the observed score with ± 9.4 for approximately 60% of the sample (see area within dotted lines, Figure 44).

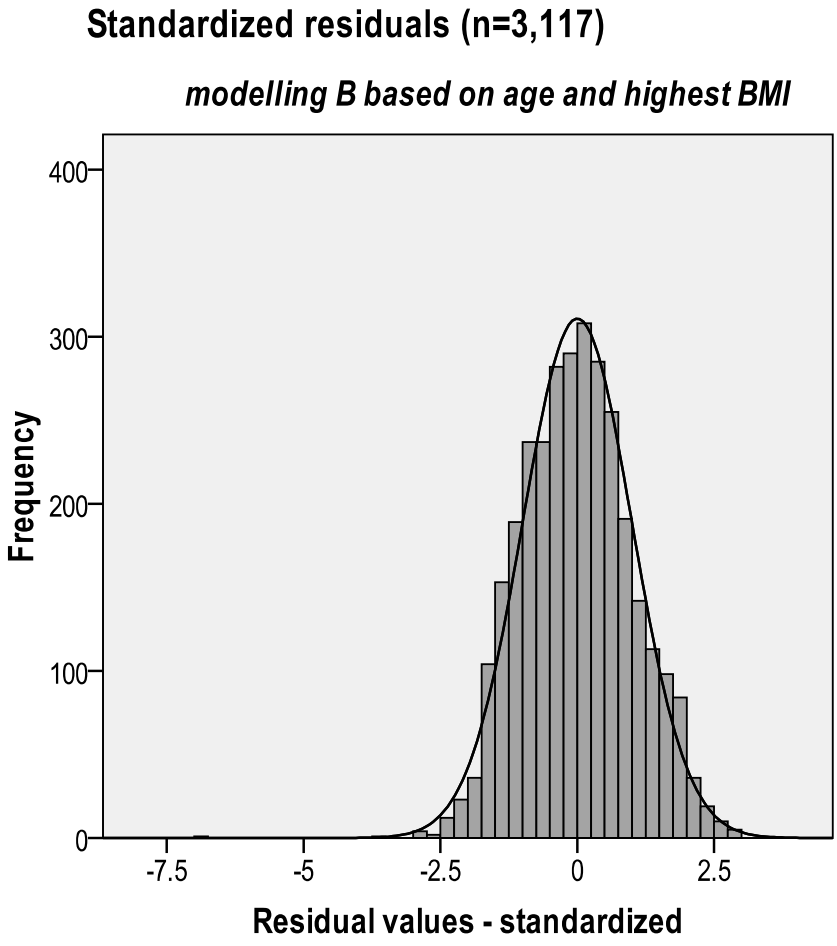

Figure 43: Distribution of standardised residuals - model B based on BMI history
The standardised residuals for the prediction of B are normally distributed.

Characteristics outliers modelling B

	General sample (n=3511)	*Stdev*	Outliers - 2 stdev (n=43)	*Stdev*	Outliers + 2 stdev (n=70)	*Stdev*
Age	57	12.9	57	11.8	55	14.4
Lowest BMI	21	3.0	23	5.1	20	2.5
Current BMI	27	5.3	32	9.0	27	6.1
Highest BMI	25	4.8	*31*	*10.0*	24	2.6
DT	17.5	6.9	*12.6*	*4.4*	***27.6***	*7.7*
B	12.2	4.9	*9.9*	*3.0*	*16.6*	*7.1*
BD	33.9	11.1	*17.7*	*8.8*	***54.8***	*2.9*

Table 40: Characteristics outliers - model B based on BMI history
Negative and positive residual value outliers stand out for having low and high scores respectively on all EDI-3 scales (most prominent differences in *bold*).

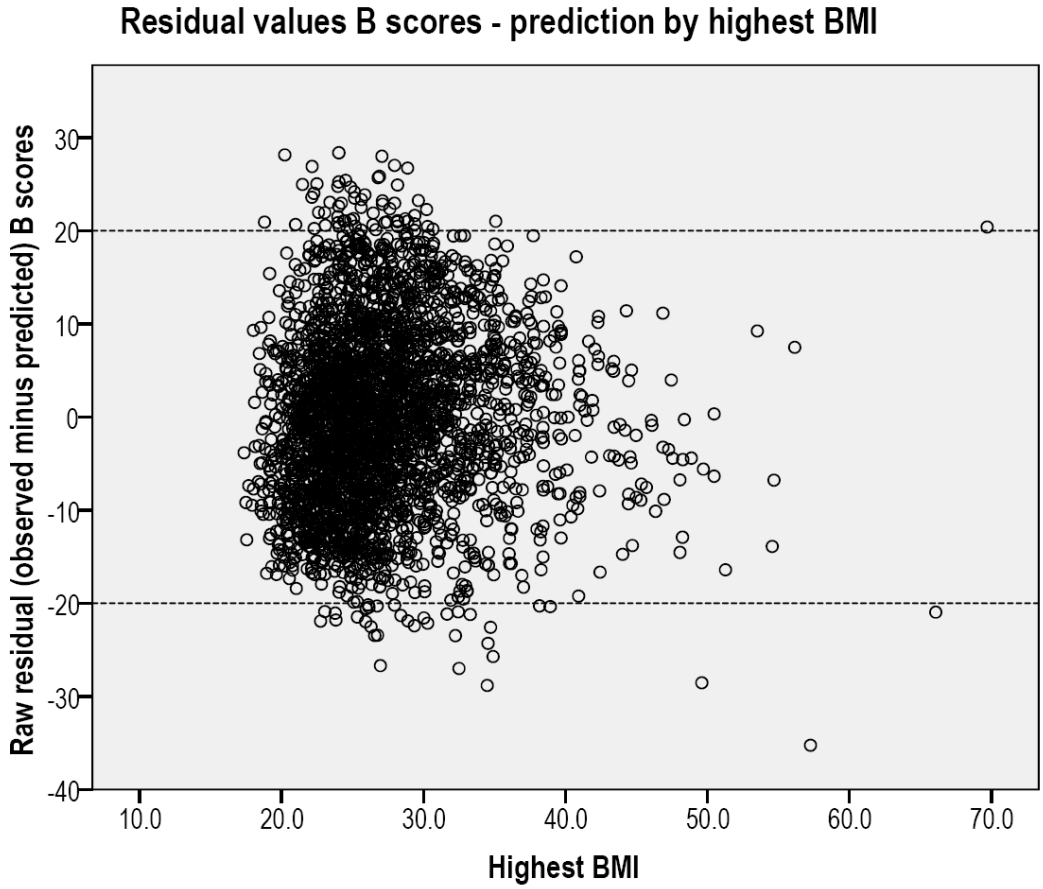

Figure 44: Raw residuals - model B based on BMI history
The residuals are equally positive and negative at each increment of the predictor variable highest BMI. Dotted lines indicate two standard deviations from the mean of the sample.

The standardised residuals of the model predicting BD are not normally distributed; as for DT there are more cases with negative residual values than predicted based on a normal distribution and there are relatively many outliers with high positive residuals (see Figure 45). The number of cases with residual values ± two standard deviations from the sample mean is not larger than 5%, as expected for an ordinary sample. The number of cases ± three standard deviations is 1.7% of the sample, which is still within 1% of what would be expected for a normally distributed sample. The characteristics of the outliers are given in Table 41; negative residual outliers have much higher mean current and highest BMI (n=8) and positive residual outliers have much higher EDI-3 scores than the rest of the sample (n=145, see Table 41). As for DT there appears

to be a subgroup of individuals for whom the model is a poor fit, Figure 46 shows that this subgroup consists of individuals with normal range current BMIs but high positive residual values; i.e. for these individuals the predicted BD score is much lower than the observed score (indicated by a dotted circle, see Figure 46). The mean BD score does significantly increase with increasing current BMI (also see Figure 35, page 131), and on average people with normal range current BMIs have low BD scores, however this does not hold true for this subgroup of individuals. Omitting this subgroup from the sample would normalise the distribution of standardised residuals and increase the predictive ability of the model for the rest of the sample.

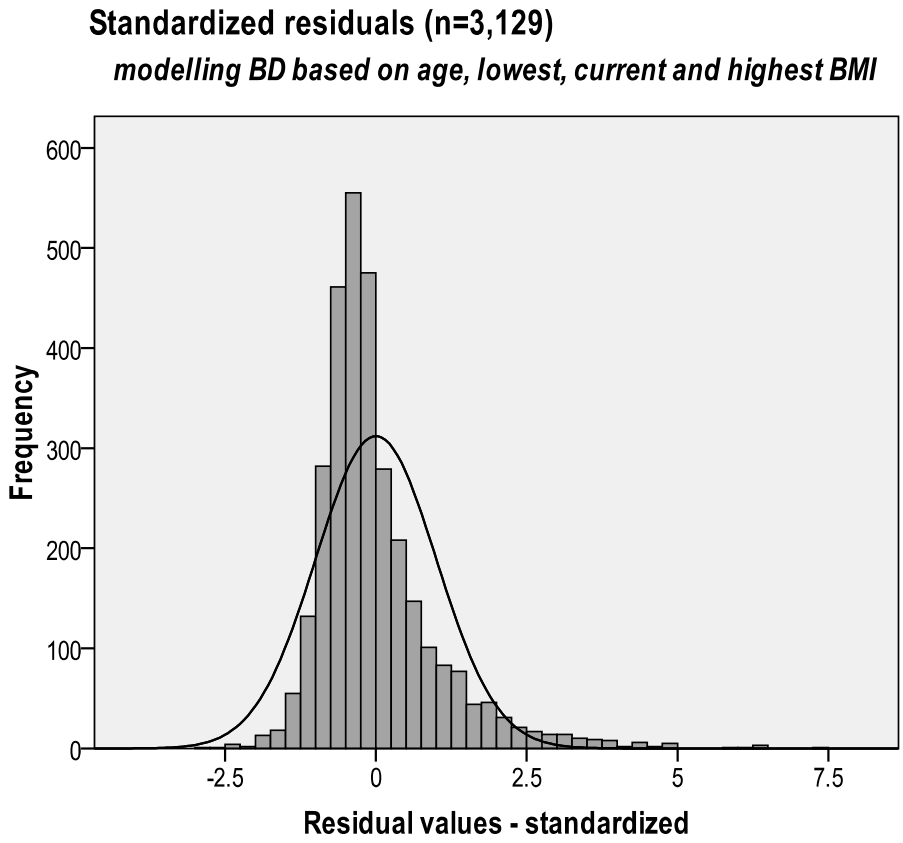

Figure 45: Distribution standardised residuals - model BD based on BMI history
The distribution of standardised residuals deviates from normality, which appears to be caused by a subgroup of individuals for whom the predicted BD score is much lower than the observed score (also see Figure 46).

Characteristics outliers modelling BD

	General sample (n=3471)	*Stdev*	Outliers - 2 stdev (n=8)	*Stdev*	Outliers + 2 stdev (n=145)	*Stdev*
Age	58	12.9	54	14.7	52	12.9
Lowest BMI	21	3.0	*31*	*9.2*	21	3.2
Current BMI	27	5.1	***54***	***11.4***	29	6.1
Highest BMI	25	4.8	***40***	***9.0***	27	5.8
DT	17.2	6.7	*24.0*	*6.2*	***28.6***	***6.8***
B	11.6	3.8	10.5	2.6	***27.4***	***5.5***
BD	33.6	11.3	*45.8*	*9.4*	*45.2*	*10.0*

Table 41: Characteristics outliers - model BD based on BMI history
Negative residual value outliers have much higher current and highest BMIs, and positive residual value outliers have much higher EDI-3 scores than the rest of the sample (most prominent differences in ***bold***).

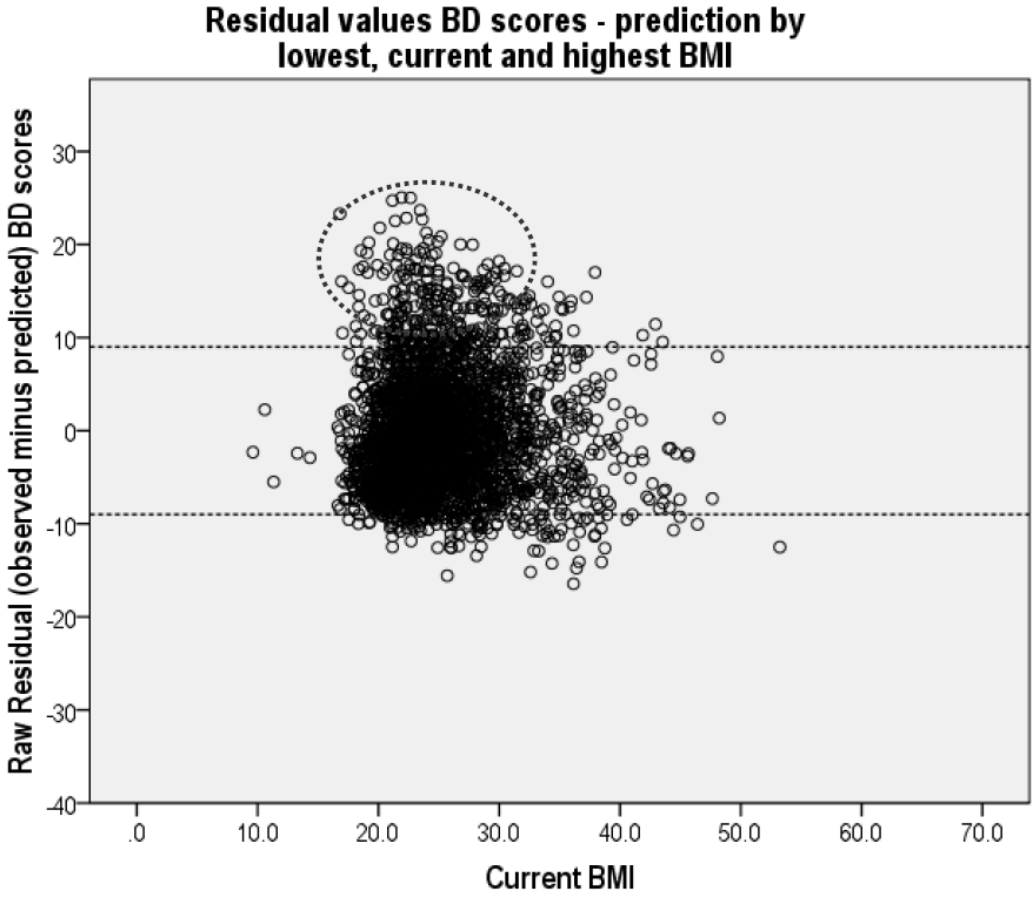

Figure 46: Raw residuals - model BD based on BMI history
Raw residuals were plotted with the predictor which had the most influence on the BD model: current BMI (beta=0.50, see Table 36, page 139). BD scores are predicted more accurately than DT and B scores (stdev raw residuals=4.5). The subgroup of individuals for whom the model is a poor fit is indicated by a dotted circle; their BD scores are underestimated.

3.6. Conclusion and discussion

3.6.1. Higher EDI scores relate to higher BMIs

This thesis confirms that EDI scores are generally higher in individuals with a higher body mass index (BMI) (Packianathan et al., 2002,Mangweth-Matzek et al., 2006,Slevec and Tiggemann, 2011). The results are not in line with the conclusion of Podar and Allik that this relationship only exists in clinical eating disorders samples, and not in general population samples (Podar and Allik, 2009). The sample size of the study by Podar and Allik is unparalleled (n = 43,722 individuals; Podar and Allik performed meta-analyses of the literature available on EDI), however the mean age of their general population sample (i.e. thus of the literature on EDI on the general population overall) was low; 19.7 years (standard deviation 3.9), and the mean BMI was also low; 21.6 (standard deviation 1.9). By comparison the mean BMI of the general population in the UK is very high; 26.8 for women (Health Survey for England, 2007). The mean BMI of the sample described in this chapter is comparable to the general UK population (also see Figure 24, page 117).

The relationship between each of the EDI Eating Disorder Risk scale scores, DT, B, and BD, and BMI are highly significant ($p < 1.00E\text{-}15$), and the correlation coefficients are moderately strong (standardised correlation coefficients (beta): 0.34, 0.32, and 0.53 for DT, B, and BD respectively). Individuals with low DT scores (below 13) have a mean BMI in the normal weight range (BMI between 20 and 25), and individuals with higher DT scores (13 and above) have a mean BMI in the overweight range (BMI above 25). The same holds true for the bulimia (B) scores; the mean BMI of individuals with low B scores (below 10) are in the normal weight range, and individuals with higher B scores (10 and above) are in the overweight range. As also indicated by the standardised correlation coefficient (beta) value, the relation with BMI is strongest for BD; individuals with

low BD scores (below 35) are in the normal weight range, individuals with moderate BD scores (between 35 and 50) are in the overweight range (BMI between 25 and 30), and individuals with high BD scores (50 and above) tend to be obese (BMI above 30). This finding that a high BMI is associatied with high BD scores is in line with the findings of Packianathan *et al*, who specifically studied the EDI scores of obese individuals; Packianathan *et al* found that their obese sample had a BD scores above the normative range of the general population, and, notably also above the normative range of the clinical eating disorders population (also see Figure 21, page 111). Individuals with missing EDI scores; because they skipped one or more questions on the questionnaire (also see paragraph 3.4.5 *Eating Disorders Inventory*, page 120), did not stand out for having very high or very low BMIs, their BMI was equal to the mean BMI of the sample.

3.6.2. BMI history predicts EDI scores better than current BMI

Highest adult lifetime BMI, rather than current BMI, was the most important predictor of DT and B scores. For BD current BMI was the most important predictor, however highest adult lifetime still explained a significant proportion of variance in BD scores when current BMI was controlled for. This result may hold important implications for the assessment of disordered eating behaviour; e.g. highest adult lifetime BMI, or more general BMI history, could be used as a proxy, or a risk factor, of disordered eating in the general population; e.g. to select individuals at risk for disordered eating in a classic two-stage study design as recommended by Hoek *et al* (Hoek and van Hoeken, 2003). Highest adult lifetime BMI is easy to assess, and individuals do not appear less willing or able to report their highest adult lifetime BMI compared to their current BMI on self-report questionnaires (data presented in Chapter 3, page 119). Whether higher EDI scores truly represent more disordered eating in individuals with higher BMIs would need to be ascertained in future studies, because most studies of the EDI

questionnaire thus far have focussed on individuals with low normal range BMIs (Podar and Allik, 2009).

3.6.3. Disordered eating in the context of BMI history

The relationship between BMI and DT, B, and BD scores did not hold true for 5% of the sample (approximately 150 out of 3,000 individuals). These individuals presented with high DT, B, and BD scores, without having, or ever having had, a high BMI. This percentage of individuals is approximately equal to the estimated prevalence of eating disorder not otherwise specified (EDNOS) in the general population (Hoek and van Hoeken, 2003,Wade et al., 2006,Machado et al., 2007,Keski-Rahkonen et al., 2007,Swanson et al., 2011), and could indicate that this group of outliers could consist of individuals at risk for eating disorders (EDs), with clinical EDs, or with a history of EDs. This finding points out that it is important to view disordered eating behaviour in the context of individual current BMI, and individual BMI history. For example, a high body dissatisfaction score could represent very different underlying behavioural and psychological traits in an individual with a normal range BMI who has never been overweight, versus an individual with a normal range BMI who has been obese in the past, versus an individual who is currently obese. Whether this subgroup of individuals with high DT, B, and BD scores, without ever having had a high BMI, truly represents a group with possible disordered eating behaviour cases would have to be ascertained through (semi-)structured clinical interviews, but it is nevertheless a finding worthy of follow-up.

3.6.4. Strengths and limitations

The strengths of the current study include: a large sample size (n = 3,624), and a sampe unselected for age and BMI as opposed to a selected cohorts (Lewis and Cachelin, 2001,Mangweth-Matzek et al., 2006), or separately sampled cohorts (Packianathan et al., 2002,Bedford and Johnson, 2006). The two most important limitations of this study are the generalisability of the EDI scores in relation to eating disorders, and the dimensionality of the EDI subscales. It remains to be

ascertained whether individuals with high EDI scores truly represent individuals with disordered eating in this sample; the results by Podar and Allik do indicate that the meaning of the scales is comparable between the general population and individuals with a clinical ED (Podar and Allik, 2009), however ideally individuals with high scores would be followed up and assessed by (semi) structured clinical interviews for eating disorders. The suggested factor structure, i.e. the subscales, of the EDI has been questioned (Garcia-Grau et al., 2010). Garcia-Grau *et al* suggest a different factor structure, e.g. by combining the drive for thinness (DT) and body dissatisfaction (BD) items; because they demonstrate that the questions of these two subscales load onto the same factor (Garcia-Grau et al., 2010). The results of this thesis support this finding, by demonstrating that DT and BD appear to explain the same variance in current BMI (Table 30). Hence, future studies may want to run analyses on observed rather than suggested factor structures.

3.6.5. Relationship between EDI scores and age

Studying the EDI scores across age groups was not the main objective of this chapter, however I nevertheless wanted to write a short comment on the results – which were presented in the Appendix (see Figure 63, Figure 64, and Figure 65, page 238). Even though EDs occur most frequently among adolescent females (also see paragraph 1.2.1 *Prevalence and incidence*, page 29) weight and shape concerns, and disordered eating are also prevalent among older women (Mangweth-Matzek et al., 2006,Slevec and Tiggemann, 2011), and specifically body dissatisfaction levels were found to be equal between younger and older age groups (Slevec and Tiggemann, 2011,Bedford and Johnson, 2006,Mangweth-Matzek et al., 2006). The current study supports this finding; even though the EDI scores significantly decrease with age (data presented in the Appendix, page 238), the correlation coefficients of these associations are very small (unstandardised coefficients are -0.081 (±0.01), -0.10 (±0.01), and -0.068 (±0.02), for DT, B, and BD respectively), indicating that the effect of age on the EDI scores

is minor. For example for BD this indicates that when age increases with one unit (one year) BD decreases with 0.068 units, thus on average women 30 years older (e.g. 50 years old versus 20 years old) score only (0.068*30=) 2.04 units lower on the BD scale (arguably negligible on a scale of 10 to 60, also see Figure 32, page 127). In line with the literature the BD scores decrease least with age (visualised in Figure 65, page 240). The small effect of age on EDI scores was significant in the current sample as a result of the large sample size, and it explains why other studies (with smaller samples) found no significant differences between the age groups; this results of this thesis are in line with the literature, concluding that age only has a minor effect on EDI scores.

Genome-wide gene analyses

4. Genome-wide gene analyses

4.1. Literature background

Genome-wide genetic studies differ from candidate gene studies in that they do not rely on hypotheses or assumptions on the underlying biological mechanism of disease, which is highly advantageous when the pathophysiology is largely unknown, as is the case in eating disorders (EDs). In genome-wide studies genetic variants (usually single nucleotide polymorphisms (SNPs)) are genotyped, which are spread throughout the genome based on knowledge of linkage disequilibrium (LD). Thanks to LD only a proportion of SNPs, 'only' approximately 500,000 SNPs, need to be genotyped to tag most (about 80%) of common genetic variation (note: rare genetic variation can not be detected). The principle and the limitations of this relatively new genome-wide association (GWA) technique were explained in more detail in paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45.

Genes are thought to be the functional units of the human genome, and disease causing genetic variants should somehow influence gene function, e.g. by affecting the level of protein expression or the protein structure (i.e. protein functionality) (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a). The fact that most diseases are multifactorial is inherent to the natural complexity of biology; all biological functions in the human body are a result of a complex interplay between many proteins (and the environment). The design of each protein is laid out in the DNA code, similarly as to how a manual describes the assembly of e.g. new furniture. Figure 47 illustrates the main aspects of protein functionality that can be affected by genetic mutations, 1) mutations in the regulatory region of the protein can affect the amount of protein that is

produced, these regulatory regions are usually located flanking the protein-coding region (generally within ± 100kb distance (Fu et al., 2011,Huang et al., 2011a)), 2) mutations in the protein-coding region can attenuate or enhance the functionality of the protein, for example, if a mutation changes the amino-acid at a key location of a receptor protein it may become insensitive to its ligand, or remain activated upon binding, hence not function normally anymore (see Figure 47).

Mutations affecting genes

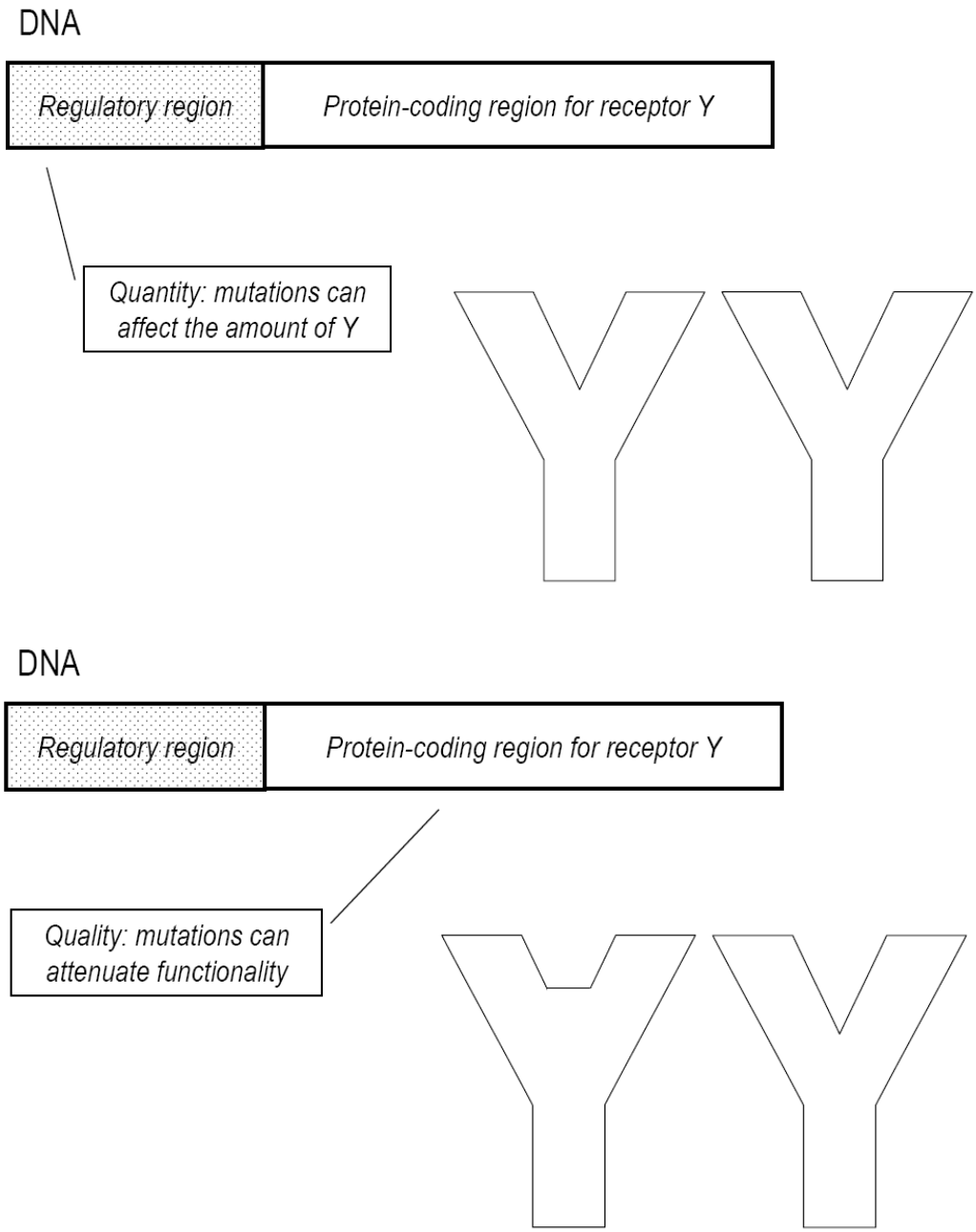

Figure 47: Causal genetic variants in relation to gene function
There are two main aspects of protein functionality that can be affected by genetic mutations; mutations in the regulatory region can affect the amount of protein that is produced (which would attenuate a biological process), and mutations in the protein-coding region, which can alter the shape and functional aspects of the protein directly, e.g. by attenuating its capacity to bind a ligand, or to transmit a signal.

In the context of biological function and mechanism of disease genome-wide association (GWA) studies are only the first step, aimed at identifying genetic risk loci, which should then be followed up in secondary analyses in order to elucidate biological function, as was done elegantly in the first GWA study in 2005 by Klein and colleagues (also see paragraph 1.3.4.2 *Where it all began*, page 50, (Klein et al., 2005)). It has however proven to be very challenging to identify genetic risk variants which pass a genome-wide significance threshold, which was set at $p < 5 \times 10^{-8}$ (or 5.00E-08) in order to distinguish true positive findings from false positive findings when testing a large number of genetic variants for association (Cichon et al., 2009) (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45). The conclusions that can be drawn from these challenges hold important clues on the genetic architecture of complex traits and disorders, in that genetic risk variants underlying these traits and disorders either include many genetic risk factors each with a (very) small effect on disease risk (odds ratios in the region of 1.1 tot 1.3, i.e. increasing the risk of carriers by 10 to 30% compared to the risk in non-carriers (Cichon et al., 2009,Cantor et al., 2010)), or they result from rare genetic variants present in less than 1% of the population. The effect of rare genetic variants on disease can not be assessed yet using current genotyping methods (also see paragraph 1.3.4.3 *Undetected heritability and genetic architecture*, page 52).

Genetic risk variants underlying disease may have effect sizes too small to detect without assessing their aggregation into genes and pathways. Because of stringent genome-wide significance threshold thousands of SNPs with "suggestive" p values are ignored, but they may nevertheless represent true associations (Cantor et al., 2010,Lehne et al., 2011). Secondary analyses such as genome-wide gene and genome-wide pathway analyses aim to address this limitation of GWA studies. Several methods have been developed for this purpose in the past couple of years (reviewed by (Cantor et al., 2010) and by (Wang et al., 2010)), but as both reviews describe, pathway analyses are

currently still in their infancy. Cantor *et al* (Cantor et al., 2010) point out critical factors for secondary analyses, of which arguably the most important is the choice of the appropriate database(s) for genes and pathways; there is a growing list of pathways that are at varying stages of completion; a notable example of a newly discovered gene and pathway is FTO in the regulation of eating behaviour (Frayling et al., 2007) (also see paragraph 1.1 Normal regulation of eating behaviour, page 20). Interestingly, and worryingly, Elbers *et al* found that the results of pathway analyses reflect the difference in pathway database information, and database organisation (Elbers et al., 2009). Because of these as yet unresolved issues Cantor *et al* (Cantor et al., 2010) recommend full transparency on the choices made when running secondary genome-wide analyses, preferably more than one database (and method) are used, and any results should be interpreted in a context-specific manner. The authors note that it is too early to set fixed guidelines for these analyses; any strict rules would hamper scientific creativity, and more time is needed before evidence-based guidelines can be developed further and applied (Cantor et al., 2010).

4.2. Aim and outline

Genome-wide association (GWA) studies on single nucleotide polymorphisms (SNPs) are the first step in identifying genetic risk loci. SNPs are however only 'tags' of possible genetic risk variants for disease (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45). Genes are thought to be the functional units of the human genome, and disease causing genetic variants should somehow influence gene function. Because biological processes are coordinated by many different proteins, acting together in pathways, many different genetic mutations could theoretically cause the same disease. Moreover, genetic risk variants underlying disease may have effect sizes too small to detect without assessing their aggregation into pathways (Cantor et al., 2010). For these reasons this chapter focusses on secondary genome-wide analyses of candidate quantitative traits of eating disorders, including genome-

wide gene and pathway analyses. Genome-wide secondary analyses are currently still in their infancy, hence two distinct methods were used. The aim of this chapter was identify genes associated with drive for thinness, bulimia, and body dissatisfaction as assessed by the Eating Disorder Inventory (EDI) (Garner, 2004), as well as to appraise the value of secondary analyses over primary genome-wide SNP analyses.

Note (also see 'Statement of work'): The author participated in an ongoing collaborative project with the Wellcome Trust Sanger Institute (Cambridge), and the UK Adult Twin Registry (TwinsUK). The collaboration included multiple research lines; the author was the lead analyst for the genome-wide association gene (GWAG) analyses. The results of three different GWAG analyses will be presented in this chapter. These results are post-hoc to the GWA SNP analyses, which were led by Dr. Vesna Boraska (Sanger Institute) and have now been submitted for publication (Boraska et al, submitted).

4.3. Data description

A subgroup of the sample presented in Chapter 3, page 113, recruited by the St Thomas' UK Adult Twin Registry (TwinsUK) took part in this study. Only twins who had consented to genetic analyses and completed the EDI questions were included in this study (also see paragraph 3.4.5 *Eating Disorders Inventory*, page 120). EDI-2 scoring was used in order to be able to replicate the study by Keski-Rahkonen *et al* (Keski-Rahkonen et al., 2005). Compared to the results of the previous Chapter, where EDI-3 was used, the question "When I am upset, I worry that I will start eating" was deleted from the bulimia (B) sub scale and the question "I feel bloated after eating a normal meal" was deleted from the body dissatisfaction (BD) sub scale. In addition the question "I like the shape of my buttocks" was deleted from the BD sub scale since this question had not been included by Keski-Rahkonen *et al* (personal communication; this BD score was labelled as Finnish-BD in Table 43); it was decided to follow the Finnish study

protocol exactly for replication purposes. Cases with missing answers to EDI questions were excluded, since missing answers would artificially lower the scale score and because missingness was not random (see paragraph 3.4.5, page 120).

To normalize the distribution of drive for thinness (DT) scores a 1/sqrt transformation was applied (the transformed DT score equals: one divided by the square root of the original DT score), BD scores were not transformed, and both traits were analysed as quantitative traits with continuous scoring. B was analysed as a binary trait because the distribution could not be normalised since there were very few individuals with elevated scores; the 75% percentile point, 12, was set as the cut-off point. Note: this is not a clinical cut-off point for Bulimia. The distribution of B scores was presented in Chapter 3, Figure 31, page 126 (note: the mean B score presented there is slightly higher since EDI-3 scores were presented in that chapter, whereas the current chapter used EDI-2 scores, also see Table 43). The subgroup of individuals who consented to genetic analyses are not different from the general sample (see Table 42, Table 43, and paragraph 3.4 *Data description*, page 113), therefore only the distribution of the DT scores will be presented here as it was transformed prior to genetic analyses (see Figure 48).

Sample overview genetic analyses

Trait	*Continuous scoring (n)*	*Case (n)*	:	*Control (n)*	*Total (n)*
DT	1934	-	:	-	*1934*
BD	1883	-	:	-	*1883*
B	-	778	:	1172	*1950*

Table 42: Sample overview genome-wide genetic analyses of the EDI
DT and BD subscales were analyses as a continuous trait, whereas the B subscale was dichotomised with individuals scoring above the 75% percentile (score 12 and above) labelled as cases.

Subgroup with consent for genetic analyses (Chapter 4)				General sample (Chapter 3)	
	Mean	*Stdev*	*Percentile 75%*	*Mean*	*Stdev*
Age	59	12.6	67	57	12.9
Current BMI	25.4	4.8	27.6	25.5	4.9
Lowest BMI	20.7	3.1	22.3	20.7	3.1
Highest BMI	27.1	5.2	29.5	27.2	5.4
EDI-2 DT	17	7.0	21	18	7.1
EDI-2 B	11	4.1	**12**	11	4.2
Finnish BD	27	10.0	34	28	10.0

EDI-3 scoring of subgroup Chapter 4 (for comparison with Chapter 3)					
	Mean	*Stdev*	*Percentile 75%*	*Mean*	*Stdev*
EDI-3 DT	17	7.0	21	18	7.1
EDI-3 B	12	4.7	13	12	4.9
EDI-3 BD	34	11.5	41	34	11.5

Table 43: Data description of the sample for genome-wide genetic analyses
Individuals who consented to genetic analyses were not different from the general sample; the latter was described in Chapter 3, page 113. B was analysed as a dichotomous trait with the 75% percentile as a cut-off between cases and controls (EDI-2 B: 12 and above, in **bold**). In Chapter 3 EDI-3 scoring was used, and in Chapter 4 EDI-2 scoring was used, hence both scoring results are presented for comparison.

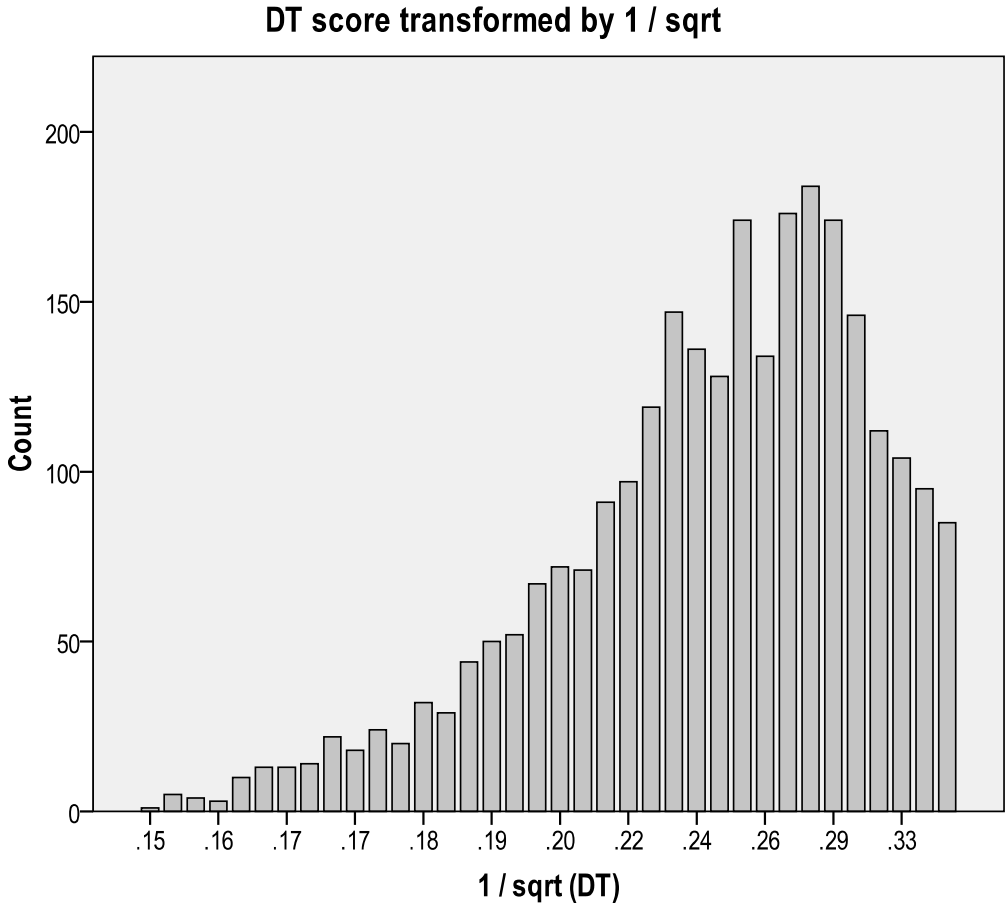

Figure 48: Distribution of DT score - transformation 1/sqrt (DT)
Since the DT scores were not normally distributed (also see Figure 30, page 125) a 1/sqrt transformation was applied (the transformed DT score equals: one divided by the square root of the original DT score).

4.4. Methods

4.4.1. Genotyping platforms, and quality control

Genotyping was conducted on three different Illumina arrays (317K, 610K, and 1M). Analyses were conducted on the merged dataset and only on overlapping SNPs (n=283,744 SNPs). Quality control (QC) was applied to each dataset separately, and further QC was applied to the merged dataset (the quality control criteria are listed in Table 44). Alleles of the three datasets were aligned to HapMap2 or HapMap3 forward strand alleles, in order to make sure the annotation of the minor allele was equal across datasets. Since spurious

associations could result from including individuals from different ancestries, a principal component analysis was performed comparing the genotypes of the TwinsUK sample to HapMap3 populations; for the HapMap project several distinct ethnic populations were genotyped, and the TwinsUK population should match the Utah residents with Northern and Western European ancestry (CEU population, The International HapMap project (HapMap, 2003)). Figure 49 shows that the TwinsUK sample indeed clusters with the CEU population (black and green data points, see Figure 49).

Quality control criteria

Subject quality control (per individual)

(i) sample call rate <98%
(ii) heterozygosity across all SNPs ≥2 standard deviations from the sample mean
(iii) evidence of non-European ancestry as assessed by PCA comparison with HapMap3 populations
(iv) observed pairwise Identity By Descent (IBD) probabilities suggestive of sample identity errors
(v) correction for misclassified monozygotic and dizygotic twins based on IBD probabilities

SNP quality control (per SNP)

(i) Hardy-Weinberg p-value<10^{-6}, assessed in a set of unrelated samples
(ii) Minor Allele Frequency (MAF) <1%, assessed in a set of unrelated samples
(iii) SNP call rate < 99% (SNPs with MAF ≥ 1% < 5%)
(iv) SNP call rate < 97% (SNPs with MAF ≥ 5%)

Table 44: Quality control (QC) criteria genome-wide genetic analyses

Subjects were excluded (i) when their DNA was of insufficient quantity or quality, (ii) when there was a risk of DNA contamination assessed by the average heterozygosity per individual; on average individuals are heterozygote for 30% of genome-wide genetic variations, (iii) when there was evidence of deviant ancestry assessed by Principal Component Analysis (PCA) comparison with individuals from various ethnicities (The International HapMap project (HapMap, 2003) (also see Figure 49), (iv) when there was evidence of relatedness or sample identity errors (e.g. duplications) assessed by pairwise Identity By Descent (IBD) probabilities, and (v) when the reported zygosity of twins was not confirmed by IBD probabilities, which could be indicative of sample identity errors. SNPs were excluded (i) when their genotype distribution in the sample violated the Hardy Weinberg Equilibrium (indicative of a technical genotyping error, also see Chapter 2, paragraph 2.5.3 *Reference population, and Hardy Weinberg Equilibrium*, page 82), (ii) when their Minor Allele Frequency (MAF), i.e. the frequency of the least frequent allele, was below 1% in the sample; quality control is more stringent for very rare alleles in order to reduce the risk of spurious results, (iii) when the SNP call rate (an indication of technical genotyping quality) was below 99% for rare SNPs, and (iv) when the SNP call rate was below 97% for common SNPs.

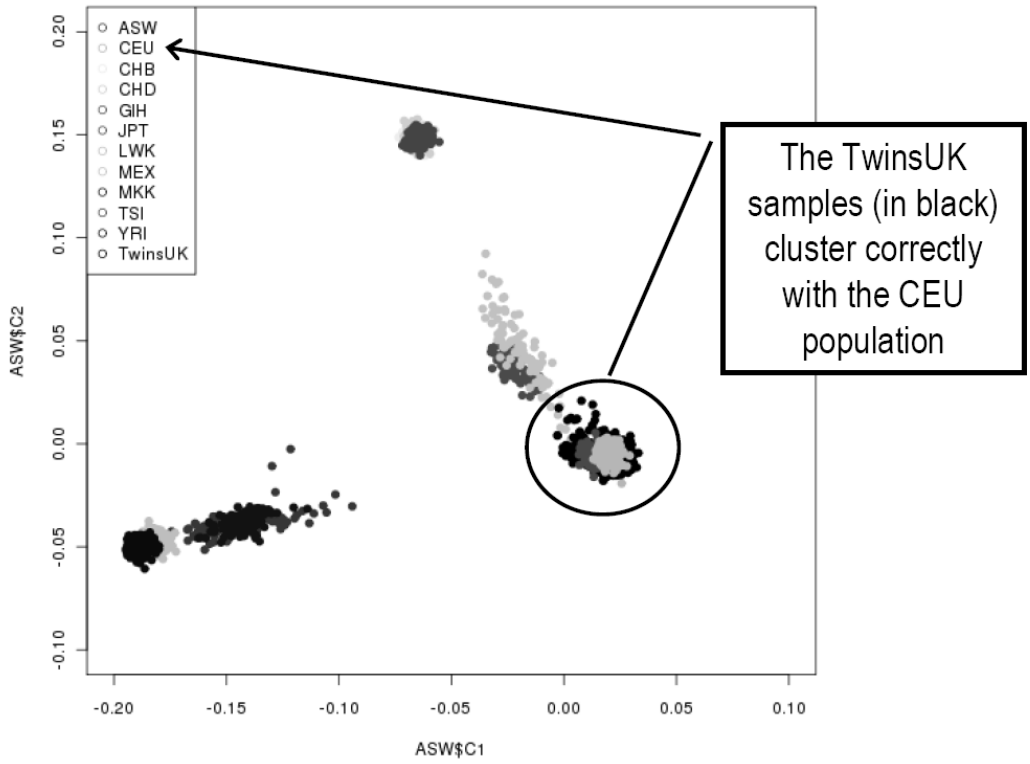

Figure 49: Principal Component Analysis (PCA) plot - evidence of ancestry
The colour printed plot is presented in the Appendix (see Figure 75, page 257). The genotypes of the individuals from the TwinsUK sample were plotted against samples from various ethnicities. The black dots represent the individuals from TwinsUK; they cluster well with the CEU population; who have Northern and Western European ancestry (The International HapMap project (HapMap, 2003)).

4.4.2. Genome-wide SNP analyses

The author was the lead analyst for the genome-wide gene (GWAG) analyses; which were post-hoc analyses of the genome-wide association (GWA) SNP analyses, which were led by Dr. Vesna Boraska (Sanger Institute). We tested 283,744 directly typed overlapping SNPs for association with three EDI subscales (DT, B, and BD) separately using the R software package GenABEL; since the sample was a twin sample all analyses were adjusted for family relatedness (Aulchenko et al., 2007,Chen and Abecasis, 2007) (Boraska, Helder, *et al*, submitted). Association analyses were restricted to individuals with complete phenotype information: 1,934 individuals for DT, 2,024 for B, and 1,915 for BD (also see Table 42, page 160). The results of the GWA SNP analyses will only be

presented briefly in this Chapter for two reasons: because the GWAG analyses are post-hoc it is important to be able to assess the quality of the initial analyses, and because the main aim of this Chapter was to appraise the added value of GWAG analyses compared to GWA SNP analyses.

4.4.3. Genome-wide gene analyses

Genome-wide association gene (GWAG) analyses were performed using two different methods; VEGAS (Liu et al., 2010) and GATES (Li et al., 2011). As was described in the literature background introduction of this chapter many different gene-based association tests have been developed; one distinguishing factor is the technical capacity of the computer systems (central processing unit (CPU) and memory) needed to run the analyses (Huang et al., 2011a,Gui et al., 2011). VEGAS and GATES are currently unique methods because a) they are fast since they do not require computationally intensive permutation procedures, b) they have no inflation of the type I error regardless of gene size and linkage disequilibrium (LD) patterns, c) they do not need raw individual genotype and phenotype data but work with SNP p values and LD information from known reference populations (HapMap, (HapMap, 2003)) and d) they are open access.

4.4.3.1. VEGAS-Sum method

The VEGAS online method defines gene boundaries as ± 50 kilo base pairs (kb) according to positions on the UCSC Genome browser hg18 assembly (note: gene boundaries could not be altered by the user), and combines SNP p values in a gene by converting them to upper tail chi-square test statistics with one degree of freedom (Liu et al., 2010). SNP p values were uploaded to the VEGAS website (http://gump.qimr.edu.au/VEGAS/) and gene p value results were obtained by email directly from the server. It is possible to include all SNPs of a gene (VEGAS-Sum method) or a subset of SNPs (e.g. only the top SNP; VEGAS-Max method). If the SNPs in the gene were completely independent of each other a combined gene test statistic, as calculated in the VEGAS-Sum method, would have a chi-square distribution with degrees of freedom equal to the number of SNPs. In

reality however most SNPs are not independent but they are in linkage disequilibrium (LD) (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45). According to Liu *et al*, ideally, LD would be taken into account by computing the gene p value through permutation, however heavy computational requirements restrict the use of permutation. Instead Liu *et al* use a Monte Carlo approach to calculate a simulated gene test statistic based on the LD structure of a set of reference individuals from the HapMap phase 2 population (Frazer et al., 2007). The simulated gene test statistic they calculate has been shown to have a distribution very similar to the observed gene test statistic under the null hypothesis; i.e. the simulated gene p value is a good approximation of the 'true' gene p-value. The simulated gene p value is the proportion of simulated test statistics that exceed the observed test statistic (Liu et al., 2010); i.e. the simulation based gene p value is the observed gene p value adjusted for LD. Liu *et al* run a number of simulations per gene, which is determined adaptively. In the first stage 10^3 simulations are performed, if the resulting simulation based p value is less than 0.1, 10^4 simulations will be performed; if the simulation based p value from 10^4 simulations is less than 0.001, 10^6 simulations will be performed (Liu et al., 2010).

4.4.3.2. GATES method

The GATES method maps SNPs onto genes according to gene coordinate information from the National Center for Biotechnology Information (NCBI). SNP p values are uploaded to the Knowledge-based mining system for Genome-wide Genetic studies (KGG) software which can be downloaded from their website (http://bioinfo1.hku.hk:13080/kggweb//home.htm, product version 2.0 was used for the current study). Gene region length was extended with 50kb on either side of the gene in order to approximate VEGAS methodology. The gene p value is equal to the most significant SNP p value of the gene after correction for the number of independent SNPs in the gene; the difference between both methods is thus that VEGAS combines the SNPs of a gene whereas GATES

corrects best SNP of the gene. GATES corrects SNP p values for the number of independent SNPs in the gene using an extended Simes method (i.e. a Simes method which takes LD into account) (Li et al., 2011). The original Simes test was proposed by RJ Simes in 1986 as a modified Bonferroni correction for multiple testing; rather than using α/n as a threshold for significance Simes used *j* * α/n as a threshold, with *j* being the ranking number of the p value when p values have been ordered (Simes, 1986). This way the correction of the most significant p value is equal to Bonferroni correction (*j* = 1), but the correction of the remaining p values is less conservative than Bonferroni correction (Simes, 1986). The type I error rate is equal to α and the Simes test is advantageous over Bonferroni when several highly correlated tests are performed (Li et al., 2011,Simes, 1986,Sarkar, 1997). In the GATES method the following formula is used:

$$\text{Gene p-value} = \text{Min}\left(\text{Me } P(j) / \text{Me } (j)\right)$$

With Min indicating that the lowest SNP p value of the gene becomes the gene p value; Me being the number of independent SNPs (i.e. only SNPs with pairwise LD below the threshold) in the gene; P(j) the p value of the j^{th} most significant SNP in the gene; Me (j) the number of independent p values among the top j SNPs (Li et al., 2011).

LD information is obtained from an appropriate reference population from the International HapMap project (CEU population was used for the current study, (HapMap, 2003)), and the user can define the threshold of LD (r-square) above which SNPs are considered connected i.e. dependent. For the current study SNPs with r-square values above the (default) threshold of 0.9 were considered dependent; this is a very conservative threshold. Lowering the threshold would decrease the number of independent SNPs per gene; the best SNP of the gene would thus be corrected for fewer independent SNPs, i.e. it would thus be corrected less conservatively. Li *et al* demonstrate that their extended Simes method has more power than the VEGAS-Max test (Li et al., 2011). The GATES method has however less power in the case of genes with multiple independent disease-susceptibility loci and relatively few neutral SNPs, in which case the VEGAS-Sum method is superior (Li et al., 2011).

In summary it can be concluded that the main differences between the VEGAS and GATES methodologies are 1) VEGAS combines SNP test results per gene whereas GATES corrects the best SNP of a gene, and 2) VEGAS accounts for LD by simulation whereas GATES uses an LD threshold above which SNPs are considered dependent. The preferred method for detecting genes with one or a few disease-susceptibility loci and relatively many neutral SNPs is the GATES method (Li et al., 2011), and for detecting genes with multiple disease-susceptibility loci it is the VEGAS-Sum method (Liu et al., 2010,Li et al., 2011). Since the genetic architecture of eating disorders is still largely unknown (also see Chapter 1 paragraph 1.3.4 *Genome-wide association (GWA) studies*, page 44), both methods were used for analyses (see Figure 50 for an overview of analyses).

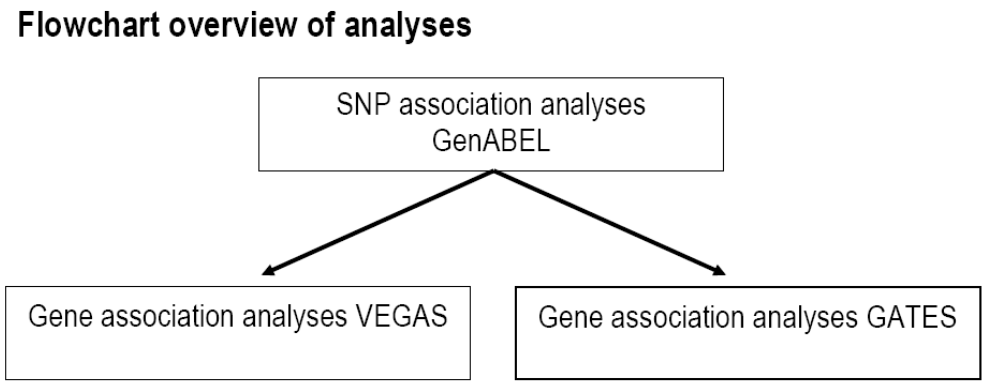

Figure 50: Flowchart overview of analyses
Gene based p values were calculated from SNP p values using two different methods; VEGAS (Liu et al., 2010) and GATES (Li et al., 2011).

4.4.4. A note on genome-wide pathway analyses

Using the GATES methodology pathway analyses were also run on these data. The GATES pathway association method (Gui et al., 2011) (also referred to as GATES-Simes) calculates a pathway p value similarly as to how a gene p value is calculated (see paragraph 4.4.3.2 *GATES method*, page 166); namely by correcting the best gene p value of a pathway for the number of genes in the

pathway (Gui et al., 2011). Using the GATES software tool, Knowledge-based mining system for Genome-wide Genetic studies (KGG), pathway p values can also be calculated using a hypergeometric method; a distinct method (not specifically developed for genetic studies) which tests the probability of by-chance enrichment of pathways by significant genes. The hypergeometric-method (referred to as GATES-Hyper (Gui et al., 2011)) calculates a cumulative probability based on the population size (i.e. the number of genes; n = 30.204), the number of successes in the population (i.e. the number of genes with a p value < 0.05), the sample size (i.e. number of genes in the pathway of interest), and the number of successes in the sample (i.e. the number of genes in the pathway with a p value <0.05).

A major limitation of these methods for pathway analyses (and of pathway analyses in general, while they are still under development (Cantor et al., 2010,Lehne et al., 2011) is that they assume that genes are independent of each other, i.e. that there is no LD between genes. In reality this is not true; hence pathway associatons need to be carefully examined in the context of LD between the genes of the pathways (note: the GATES pathway methods are currently being updated and improved (personal communication with Miaoxin Li (Li et al., 2011)). Because of the limitations of the methods used it was decided not to present the results of the pathway analyses in the chapter, but in the Appendix instead; the limitations were discussed in further detail in the discussion and conclusion of this chapter (see paragraph 4.8, page 190). The top pathways for drive for thinness (DT) were presented in the Appendix (see Table 66 and Table 67, page 250).

4.5. *Results Drive for Thinness*

4.5.1. Results genome-wide association SNP analysis

The top SNP associated with drive for thiness (DT) scores is rs1436174 with a p value of 2.31E-06, this SNP is located on chromosome one within 50kb of the gene cornichon homolog-3 (CNIH3). General note: gene names are listed alphabetically in the Appendix (see Table 61 to Table 65, page 244). None of the SNP p values pass a Bonferroni correction for multiple SNP testing (0.05 / 283,744 SNPs = 1.76E-07), nor do they pass an arbitrary Bonferroni threshold for suggestive association: with α set at half the chance (α=0.5) a SNP is associated with the phenotype (0.5 / 283,744 SNPs = 1.76E-06). Figure 51 shows a plot of the SNP p values (ordered by chromosome and position in the chromosome), and the quantile-quantile (QQ)-plot which is a quality control measure for genome-wide association studies; observed SNP p values are plotted against an equal number of normally distributed p values. When running a large number of statistical tests (283,744 SNPs were tested for association with DT scores) the resulting p values will be relatively uniformly distributed, which is what is expected under the null hypothesis of no association with the phenotype. In the case of true association there would be more SNPs strongly associated to the phenotype than expected by chance; i.e. the only deviation from uniformity would be expected at the lowest p values. In general the QQ-plot should thus show a straight diagonal line; it provides evidence of no systematic over-estimation of the association signal due to confounders, e.g., population structure or genotyping error. This is the case for DT (see Figure 51), which is reassuring of the quality of the QC and GWA SNP analyses.

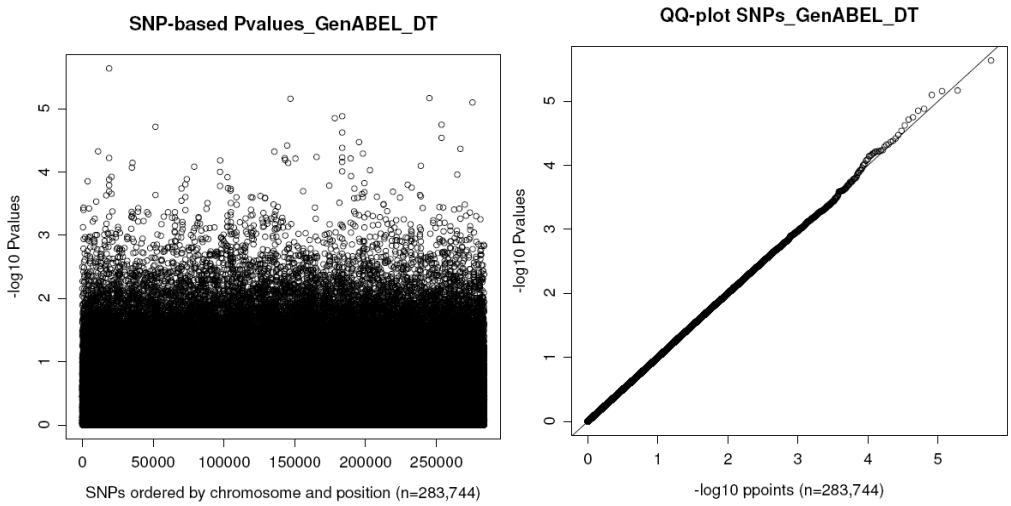

Figure 51: P value and QQ plot - SNP association results DT
Note: p values were plotted after –log10 transformation: a p value of 0.05 is thus plotted at –log(0.05,10)= 1.30. The top SNP, with a p value of 2.31E-06, is plotted at 5.64 (upper left corner of the p value plot). The QQ-plot shows that the p values do not deviate from the diagonal, indicating that the p values are generally uniformly distributed. Plots were generated using R software, the 'ppoints' function was used to generate 283,744 normally distributed probability points.

4.5.2. Results genome-wide association gene analysis - DT

The top gene associated with drive for thinness (DT) scores according to the VEGAS-Sum method is brain-derived neurotrophic factor (BDNF) with a p value of 3.70E-05 (see Table 45). Using the VEGAS (Liu et al., 2010) definition of genes (the gene region plus SNPs ± 50kb) 23 SNPs are located in the BDNF gene, 11 out of 23 SNPs have a p value below 0.01 (see Table 46, in **bold**). Out of these 11 SNPs four SNPs are in moderate LD with the strongest associated SNP (pairwise LD between 0.6 and 0.8, see Figure 52). The second most significant SNP is however not in strong LD with the top SNP (rs6265, LD < 0.6, see Table 46 and Figure 52), interestingly this SNP is a missense variation; the two different alleles, G and A, lead to two different amino acids, Valine (Val) and Methionine (Met), in the BDNF protein, i.e. they lead to a different protein structure of BDNF (Noble et al., 2011,Brandys et al., 2011). BDNF is the only gene in the top 25 genes of the VEGAS method also present in the top 25 of the GATES method for gene association (BDNF ranked 7th in GATES, see Table 47). The BDNF gene p value does however not pass Bonferroni correction for the number of genes tested

(0.05/17,555 = 2.85E-06), nor does it pass the threshold for suggestive association (0.5/17,555 = 2.85E-05). Table 45 also shows the top SNP per gene (according to NCBI annotation), and its rank among SNPs; the top SNP of BDNF was rs7116850, which was the fifth most significant SNP overall (see Table 45). Several genes share the same top SNP; there are only 19 unique top SNPs among the top 25 genes. The SNP rs756441 is an example of this; it is the top SNP of three different genes (see Table 45 in grey); these genes are located very closely together and the overlap is a result of defining gene regions as ± 50kb on either side (see Figure 53). The ranking of the top SNPs of the top genes (most right column in Table 45) shows that most genes would not have been picked up without the VEGAS method, e.g. if only the top SNPs (e.g. the top 250 SNPs) had been examined further.

Results VEGAS method - DT

Rank	*Rank in GATES*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	***7***	***BDNF***	***11***	***23***	***3.70E-05***	rs7116850	5
2	40	KRT8	12	11	1.50E-04	rs2638511	42
3	607	ANKRD5	20	31	3.29E-04	rs725565	333
4	50	RXRB	6	21	5.66E-04	rs756441	52
5	32	SLC39A7	6	18	7.73E-04	rs756441	52
6	#N/A	GOLGA6B	15	1	8.28E-04	-	-
7	433	SLC4A9	5	8	1.02E-03	rs1862176	1296
8	44	BBS4	15	6	1.18E-03	rs730180	224
9	105	COL11A2	6	37	1.22E-03	rs756441	52
10	72	KRT78	12	23	1.23E-03	rs2638511	42
11	151	PRMT5	14	10	1.25E-03	rs11623624	339
12	49	NDUFA12	12	19	1.27E-03	rs12368216	32
13	441	HBEGF	5	7	1.50E-03	rs7268	988
14	13173	UBE2S	19	10	1.82E-03	rs6509940	52624
15	136	SLC35A4	5	7	1.90E-03	rs250429	503
16	371	FAM84B	8	11	1.95E-03	rs7839958	694
17	123	RBM23	14	12	1.95E-03	rs2295682	169
18	#N/A	C14orf94	14	11	1.96E-03	-	-
19	536	APBB3	5	7	1.97E-03	rs1835148	1812
20	215	MTA3	2	10	2.06E-03	rs4953636	242
21	238	REM2	14	11	2.19E-03	rs11623624	339
22	440	ASS1	9	45	2.23E-03	rs12375699	144
23	771	ANKHD1-EIF4EBP3	5	10	2.29E-03	rs10042299	2857
24	195	ANKHD1	5	9	2.31E-03	rs1432959	602
25	643	EIF4EBP3	5	7	2.33E-03	rs1864255	5507

Table 45: Results VEGAS method - Top 25 genes DT
BDNF is the only gene which is also present in the top 25 of the GATES method (rank 7, in ***bold***). BDNF is located on chromosome (Chr) 11, and has 23 SNPs (nSNPs). Pvalue is the gene p value uncorrected for the number of genes tested (none of the genes pass a

Bonferroni correction for multiple testing). The 'Top SNP' is the top SNP of the gene, and the 'Rank SNP' is its rank among SNPs overall. Some genes share a top SNP, rs756441 is an example of this (in grey, also see Figure 53). Two genes were not part of the GATES gene set (indicated by #N/A).

Gene	*SNP*	*Position*	*Risk allele*	*Frequency*	*Type of variation*	*SNP p value*
BDNF	rs10767652	27628826	G	0.48	-	6.49E-01
-	rs7939810	27633409	C	0.36	-	**3.25E-03**
-	rs1387144	27635319	C	0.41	-	**7.02E-04**
-	rs7116850	27645530	G	0.28	-	**1.31E-05**
-	rs4074134	27647285	T	0.21	-	**5.91E-05**
-	rs4923461	27656910	G	0.21	-	**6.94E-05**
-	rs925946	27667202	T	0.32	-	1.96E-02
-	rs10501087	27670108	C	0.21	-	**4.16E-05**
-	rs2203877	27670910	C	0.46	-	1.75E-01
-	rs6265	27679916	T	0.19	missense	**2.38E-05**
-	rs11030104	27684517	G	0.21	intron	**9.79E-05**
-	rs10835211	27701365	A	0.26	intron	**5.76E-03**
-	rs7934165	27731983	A	0.47	intron	1.83E-01
-	rs12273363	27744859	C	0.21	-	**3.89E-03**
-	rs908867	27745764	T	0.10	-	2.98E-01
-	rs1491850	27749725	C	0.43	-	8.63E-01
-	rs1157659	27757622	G	0.48	-	2.68E-01
-	rs1008041	27772997	C	0.11	-	8.46E-01
-	rs2172229	27776622	G	0.39	-	7.33E-01
-	rs728633	27781979	G	0.24	-	**1.96E-03**
-	rs10501091	27787783	C	0.36	-	9.42E-01
-	rs10501089	27788859	T	0.04	-	1.28E-01
-	rs1491872	27792891	T	0.35	-	3.67E-01

Table 46: Details of BDNF SNPs - DT

There are 23 SNPs in the BDNF gene according to the VEGAS definition of genes (Liu et al., 2010). SNPs were ordered on position in the chromosome (BDNF is located on chromosome 11 (CHR 11)). Frequency refers to the risk allele frequency. The variation in one SNP (rs6265) is a missense variation, meaning that the two alleles of the SNP lead to two different amino acids in the BDNF protein, i.e. lead to a different protein structure of BDNF. Three SNPs are located in the intron of the gene; they are not translated into BDNF protein. BDNF has several SNPs suggestively associated to DT scores, p values below 0.01 are indicated in **bold**. Details on LD between the SNPs are given in Figure 53, page 173. BDNF is the top gene according to the VEGAS method with a p value of 3.70E-05.

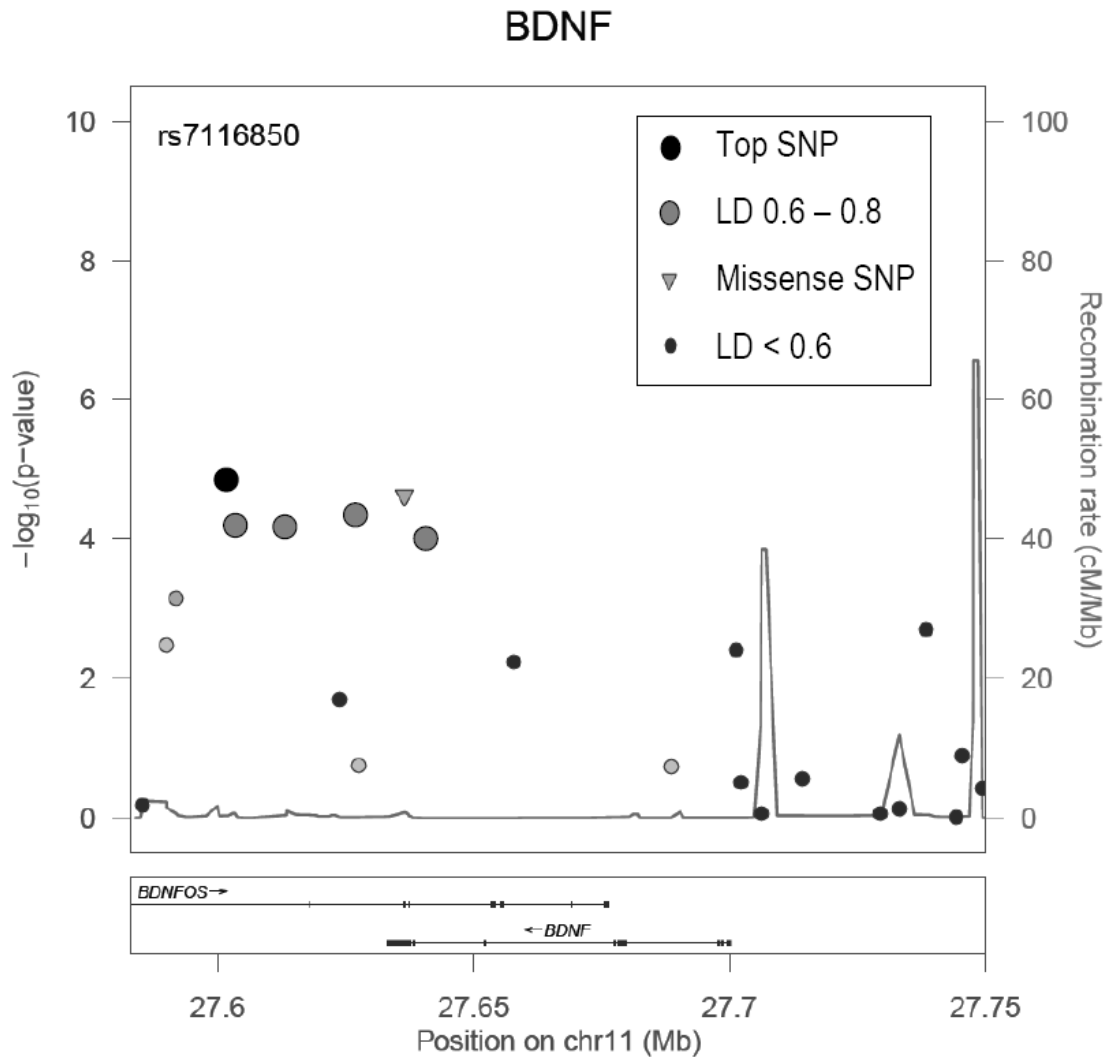

Figure 52: Plot of BDNF SNPs - DT

SNPs of BDNF (± 50kb on either side) are plotted. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate, i.e. the average rate of 'reshuffling' of parental DNA; a high recombination rate decreases the LD between SNPs. The top SNP in BDNF is rs7116850 (indicated by the large black circle). Four SNPs are in moderate LD with the top SNP (LD between 0.6 and 0.8, indicated by large grey circles); these four SNPs are also among the lowest p values of the gene. Smaller circles are SNPs in low LD with the top SNP (LD < 0.6). The missense SNP (indicated by the triangle) is also not in strong LD with the top SNP (LD < 0.6) but has the second lowest p value of the gene (also see Table 46). The plot was generated using LocusZoom (Pruim et al., 2010). An extended plot including the complete LD region (LD ≥ 0.4) of the top SNP was presented in the Appendix, page 254.

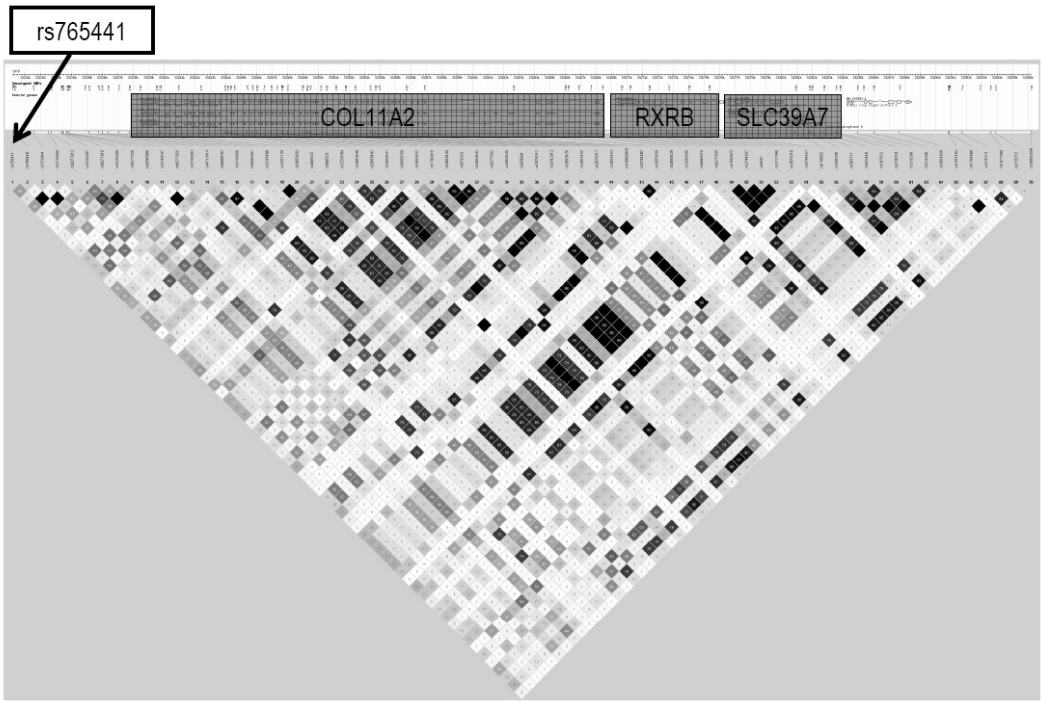

Figure 53: Example of genes with overlapping top SNP
The SNP rs765441 was the 52nd most significant SNP overall, and is the top SNP of three different genes (also see Table 45). The three genes, COL11A2, RXRB, and SLC39A7 are located closely together on chromosome six. Rs756441 is located most closely to COL11A2, but due to the gene region definition of ± 50kb it is assigned to all three genes. The image shows that the SNPs of these three genes almost completely overlap due to this definition of gene regions. The blackness of the blocks below the genes visualise the LD between the SNPs of the genes, with black blocks indicating complete LD. The image was generated using Haploview (Barrett et al., 2005).

The top gene associated with DT scores resulting from the GATES method is cornichon homolog-3 (CNIH3) with a p value of 3.82E-05 (see Table 47). The gene p values were corrected for the number of tested SNPs within the gene, but not for the number of tested genes. None of the genes pass a Bonferroni correction for the number of genes (0.05/30,204 genes = 1.66E-06), nor do they pass the threshold for suggestive association (0.5/30,204 = 1.66E-05). Note: the GATES method uses the NCBI database of genes whereas the VEGAS method uses the UCSC database (also see paragraph 4.4.3 *Genome-wide gene analyses*, page 165), hence the difference in the number of genes. The top SNPs of the genes were all among the top 65 of most significant SNPs overall. Several genes share the same top SNP; there are only 14 unique top SNPs among the top 25 genes (rs10280445 was the top SNP of eight genes, see Table 47 in grey). BDNF is the

only gene present in both the VEGAS and the GATES top 25 genes, indicating that there were several independently associated SNPs in the gene (making it a gene VEGAS would pick up) and that the top SNP was strongly associated (making it a gene GATES would pick up) (also see Table 46 and Figure 52). CNIH3 on the other hand is a gene that would only be picked up by GATES; it has 24 SNPs of which only three SNPs have a p value below 0.01, the top SNP of CNIH3 is the number one most strongly associated SNP overall (see Table 47 and Figure 54).

Results GATES method - DT

Rank	*Rank in VEGAS*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	116	CNIH3	1	24	3.82E-05	rs1436174	1
2	#N/A	RPS25P1	11	7	6.89E-05	rs7116850	5
3	921	GLP2R	17	20	1.17E-04	rs7218549	2
4	#N/A	BDNFOS	11	16	1.32E-04	rs7116850	5
5	107	RGS10	10	16	1.73E-04	rs4752325	6
6	#N/A	LOC100506140	21	30	1.89E-04	rs2830230	4
7	***1***	***BDNF***	***11***	***23***	***1.89E-04***	rs7116850	5
8	#N/A	LOC645001	19	2	2.01E-04	rs1076976	35
9	417	LYPD4	19	2	2.01E-04	rs1076976	35
10	325	PWWP2A	5	6	2.16E-04	rs7378736	25
11	#N/A	LOC100422623	19	3	2.38E-04	rs1076976	35
12	#N/A	LOC100420539	7	1	2.39E-04	rs10280445	63
13	3094	CYYR1	21	50	3.06E-04	rs2830230	4
14	#N/A	LOC100506554	9	8	3.85E-04	rs4841940	18
15	#N/A	LOC652489	7	2	3.88E-04	rs10280445	63
16	74	CEACAM3	19	5	4.45E-04	rs1076976	35
17	273	CEACAM6	19	7	4.83E-04	rs1076976	35
18	776	FABP6	5	9	5.13E-04	rs7378736	25
19	740	OSBPL1A	18	42	5.33E-04	rs2588568	7
20	#N/A	LOC100288904	7	3	6.27E-04	rs10280445	63
21	#N/A	LOC730376	7	3	6.27E-04	rs10280445	63
22	#N/A	LOC100419984	7	3	6.27E-04	rs10280445	63
23	#N/A	LOC100507269	7	3	6.27E-04	rs10280445	63
24	#N/A	LOC100128326	7	3	6.27E-04	rs10280445	63
25	#N/A	PSPHL	7	3	6.27E-04	rs10280445	63

Table 47: Results GATES method - Top 25 genes DT
CNIH3 is the top gene according to the GATES method, its top SNP is the top SNP overall (rs1436174, rank 1, upper row). BDNF is the only overlapping gene between the VEGAS and GATES methods (in ***bold***); it is the most significant gene in the VEGAS-Sum method (also see Table 45). Many genes share the same top SNP (rs10280445 is an example, in grey); there are only 14 unique top SNPs among the top 25 genes. Fourteen genes were not part of the VEGAS gene set (indicated by #N/A); the VEGAS gene set was considerably smaller than the GATES gene set (17,555 vs 30,204 genes also see 4.4.3 *Genome-wide gene analyses*, page 165).

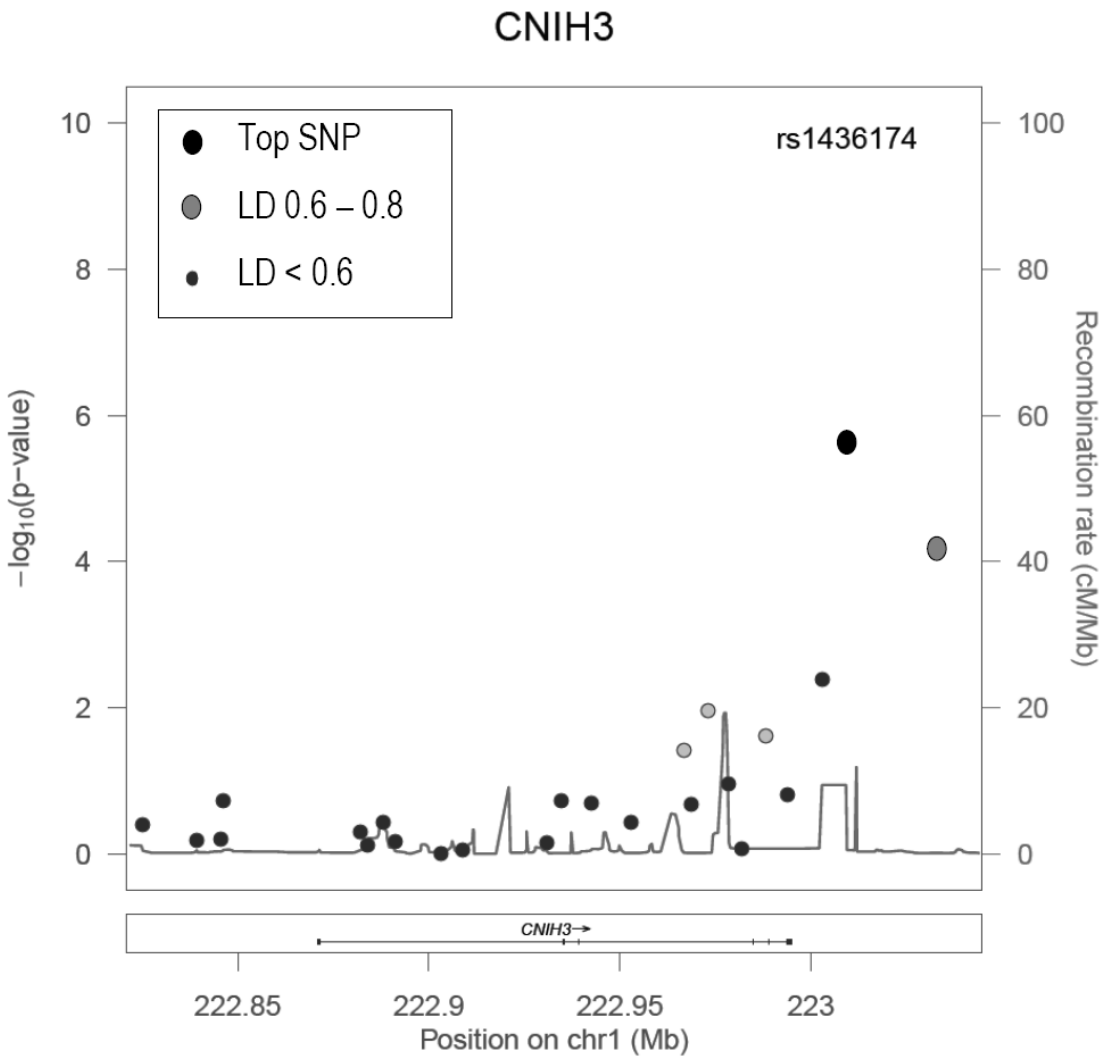

Figure 54: Plot of CNIH3 SNPs - DT

CNIH3 is a typical gene that would be picked up by the GATES method, but not by the VEGAS method. There are 24 SNPs in the gene of which only three SNPs have a p value below 0.01. Its top SNP is however the top SNP overall (rs1436174, p= 2.31E-06, indicated by the large black circle). The second most significant SNP of the gene is in moderate LD with the top SNP (indicated by the large grey circle); the other SNPs are not in LD with the top SNP (smaller circles). The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The plot was generated using LocusZoom (Pruim et al., 2010). An extended plot including the complete LD region (LD ≥ 0.4) of the top SNP was presented in the Appendix, page 255.

4.6. Results Bulimia

4.6.1. Results genome-wide association SNP analysis - B

The top SNP associated with Bulimia (B) scores is rs2895316 with a p value of 1.87E-06, this SNP is located on chromosome three within 50kb of the gene LOC100128733 (an uncharacterised or pseudo gene with no known function). General note: gene names are listed alphabetically in the Appendix (see Table 61 to Table 65, page 244). None of the SNPs associated with B pass a Bonferroni correction for multiple SNP testing, nor do they pass a Bonferroni threshold for suggestive association. Figure 55 shows a plot of the SNP p values and the QQ-plot; the p values are generally normally distributed.

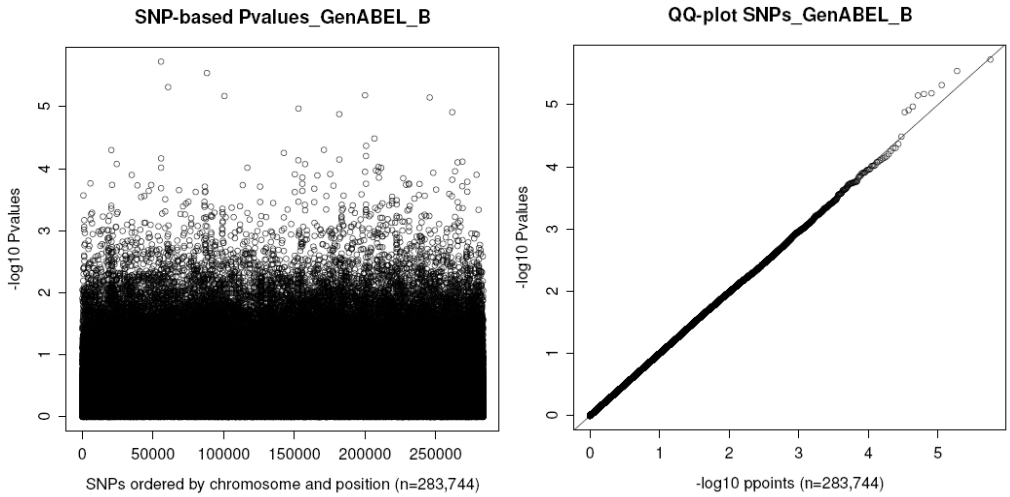

Figure 55: P value and QQ plot - SNP association results B
P values are plotted after –log10 transformation; the top SNP has a p value of 1.87E-06 and is plotted at 5.73 (upper left data point p value plot). The p values are generally normally distributed. Plots were generated using R software.

4.6.2. Results genome-wide association gene analysis – B

The top gene associated with Bulimia (B) scores according to the VEGAS-Sum method is alkylation repair homolog-3 (ALKBH3) with a p value of 7.10E-05 (see Table 48). There are 17 SNPs in this gene of which seven have a p value below 0.01; only one SNP is in strong LD with the top SNP (LD > 0.8, indicated by a square, see Figure 56). Note: the plot shows there are three more genes at this location; HSD17B12, LOC729799, and AG2. HSD17B12 ranked 2nd in the VEGAS method (see Table 8), which is expected given the defition of genes as ± 50kb; the SNPs annotated to ALKBH3 and HSD17B12 almost completely overlap.

LOC729799 was not part of the VEGAS gene set, but ranked 182nd out of 30,204 genes according to the GATES method (data not shown), and AG2 was not part of either the VEGAS or the GATES gene set. The p value of ALKBH3 does however not pass a Bonferonni threshold for multiple gene testing, nor does it pass the arbitrary threshold of suggestive association (see paragraph 4.5.1 *Results genome-wide association SNP analysis*, page 169) (0.5/17,555 = 2.85E-05). The ranking of the top SNPs of the top genes shows that most genes would not have been picked up without the VEGAS method, if only top SNPs had been examined further. Six genes were present in both the VEGAS and GATES top 25 (see Table 48 and Table 49, in ***bold***).

Results VEGAS method - B

Rank	*Rank in GATES*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	112	ALKBH3	11	17	7.10E-05	rs2434474	72
2	238	HSD17B12	11	21	1.77E-04	rs2862999	206
3	***10***	***ZNF785***	***16***	***3***	***2.21E-04***	rs9934806	46
4	54	CNOT2	12	28	2.64E-04	rs12831626	44
5	67	ZNF689	16	3	3.02E-04	rs7197475	322
6	66	C14orf39	14	4	3.40E-04	rs12436579	424
7	***23***	***RDH10***	***8***	***20***	***3.75E-04***	rs16938613	14
8	***14***	***KCNMB4***	***12***	***23***	***8.24E-04***	rs1654964	11
9	48	SIX6	14	3	1.04E-03	rs12436579	424
10	***9***	***FAM71E1***	***19***	***16***	***1.08E-03***	rs1274597	20
11	#N/A	LOC554235	19	13	1.16E-03	-	-
12	117	PTS	11	4	1.19E-03	rs919479	500
13	191	SIX1	14	3	1.19E-03	rs10483727	1159
14	290	HMGB2	4	6	1.24E-03	rs12498911	1281
15	30	JOSD2	19	14	1.25E-03	rs1298062	88
16	36	C19orf63	19	13	1.49E-03	rs1298062	88
17	240	SAP30	4	5	1.56E-03	rs7655786	537
18	1885	KIRREL	1	24	1.57E-03	rs6686246	880
19	205	C9orf103	9	17	1.62E-03	rs1052690	251
20	93	MYBPC2	19	17	1.81E-03	rs1298062	88
21	101	ZNF264	19	16	1.89E-03	rs4801447	108
22	142	RPL7	8	12	1.94E-03	rs12682469	160
23	421	SCRG1	4	8	2.02E-03	rs7655786	537
24	***1***	***HTR1A***	***5***	***5***	***2.60E-03***	rs1478498	2
25	***3***	***CCNL1***	***3***	***9***	***2.60E-03***	rs7624327	3

Table 48: Results VEGAS method – Top 25 genes B
The top gene according to the VEGAS method is ALKBH3, it ranked 112th according to the GATES method. ALKBH3 is located on chromosome (Chr) 11, there are 17 SNPs and its top SNP ranked 72nd overall. Six genes were also present in the top 25 of the GATES method (in ***bold***). Some genes are located closely together and share the same top SNP (in grey an example).

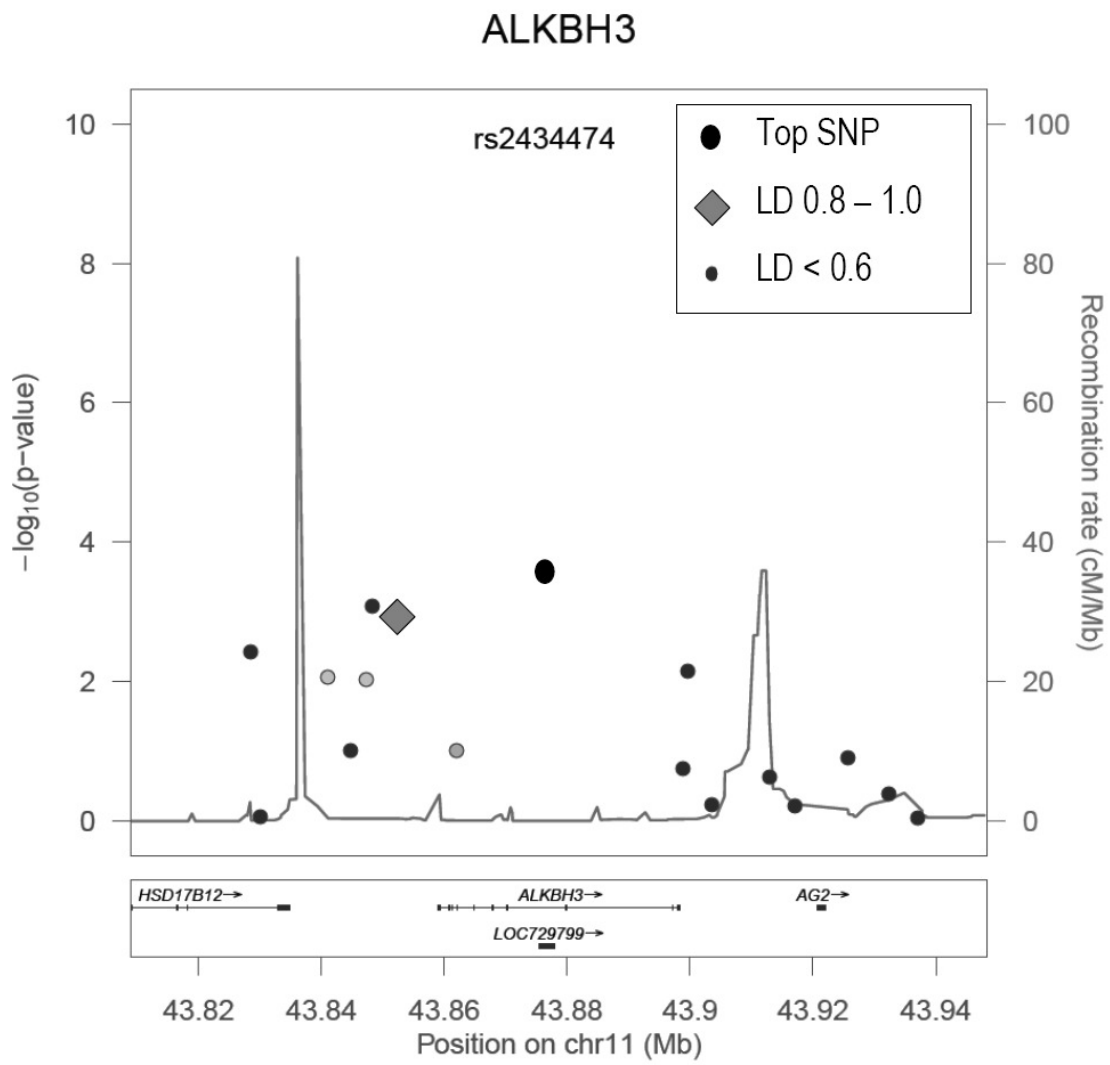

Figure 56: Plot of ALKBH3 SNPs - B
SNPs of ALKBH3 ± 50kb are plotted, the plot includes the complete LD region (LD ≥ 0.4) of the top SNP. The top SNP rs2434474 was ranked 72nd most significant SNP overall (see Table 48). Only one SNP is in strong LD with the top SNP (indicated by a square), this is the 3rd most significant SNP of the gene. Several SNPs in the gene are suggestively associated with B scores. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The plot was generated using LocusZoom (Pruim et al., 2010).

The top gene resulting from the GATES method is 5-hydroxytryptamine (serotonin) receptor 1A (HTR1A) with a p value of 1.33E-05, its top SNP was the 2nd most significant SNP overall (see Table 49). The top genes do not pass a Bonferroni correction for multiple gene testing, but HTR1A does pass the Bonferroni threshold for suggestive association (0.5/30,204 = 1.66E-05). Figure 57 shows that only one SNP of the gene has a low p value, rs1478497, which is

located approximately 40kb upstream of HTR1A (see Figure 57). Six genes were present in both the VEGAS and the GATES top 25 (see Table 49, in ***bold***).

Results GATES method - B

Rank	*Rank in VEGAS*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	***24***	***HTR1A***	***5***	***5***	***1.33E-05***	rs1478498	2
2	#N/A	KRT18P34	3	6	2.67E-05	rs7624327	3
3	***25***	***CCNL1***	***3***	***9***	***3.88E-05***	rs7624327	3
4	#N/A	LOC339894	3	13	5.39E-05	rs7624327	3
5	#N/A	LOC100498859	3	13	5.39E-05	rs7624327	3
6	#N/A	LOC100289542	12	13	5.51E-05	rs4763075	4
7	#N/A	CDRT7	17	18	1.01E-04	rs2058178	6
8	571	ZNRF4	19	19	1.91E-04	rs8111234	8
9	***10***	***FAM71E1***	***19***	***4***	***2.51E-04***	rs1274597	20
10	***3***	***ZNF785***	***16***	***2***	***3.14E-04***	rs9934806	46
11	492	TEAD1	11	43	3.99E-04	rs2727408	9
12	#N/A	LOC100506305	11	9	5.40E-04	rs7937953	17
13	#N/A	LOC100506352	11	10	6.07E-04	rs7937953	17
14	***8***	***KCNMB4***	***12***	***19***	***6.18E-04***	rs1654964	11
15	#N/A	LOC100128733	3	8	6.47E-04	rs10511259	24
16	#N/A	MIR107	10	6	6.50E-04	rs1274046	32
17	#N/A	LOC100130099	1	16	6.65E-04	rs4313384	13
18	206	RBP3	10	18	6.92E-04	rs2376635	12
19	#N/A	ZNF679	7	4	7.19E-04	rs11980921	56
20	52	KLC1	14	9	7.21E-04	rs861544	39
21	5362	HMG20B	19	3	8.09E-04	rs11085023	76
22	5475	TBXA2R	19	3	8.09E-04	rs11085023	76
23	***7***	***RDH10***	***8***	***18***	***8.23E-04***	rs16938613	14
24	#N/A	PRAMEF21	1	1	8.64E-04	rs2933080	215
25	#N/A	RPL12P4	20	9	9.17E-04	rs6127380	41

Table 49: Results GATES method – Top 25 genes B

The top gene according to the GATES method is HTR1A, it does not pass a Bonferroni correction for multiple testing but it does pass a threshold for suggestive association (also see paragraph 4.5.1, page 169) (0.5/30,204 = 1.66E-05). HTR1A is located on chromosome (Chr) five and there were five SNPs in the gene, its top SNP ranked 2nd overall. Some genes are located closely together and share the top SNP (an example in grey). Six genes were present in the top 25 of both the VEGAS and the GATES method (in ***bold***). Thirteen genes were not part of the VEGAS gene set (indicated by #N/A).

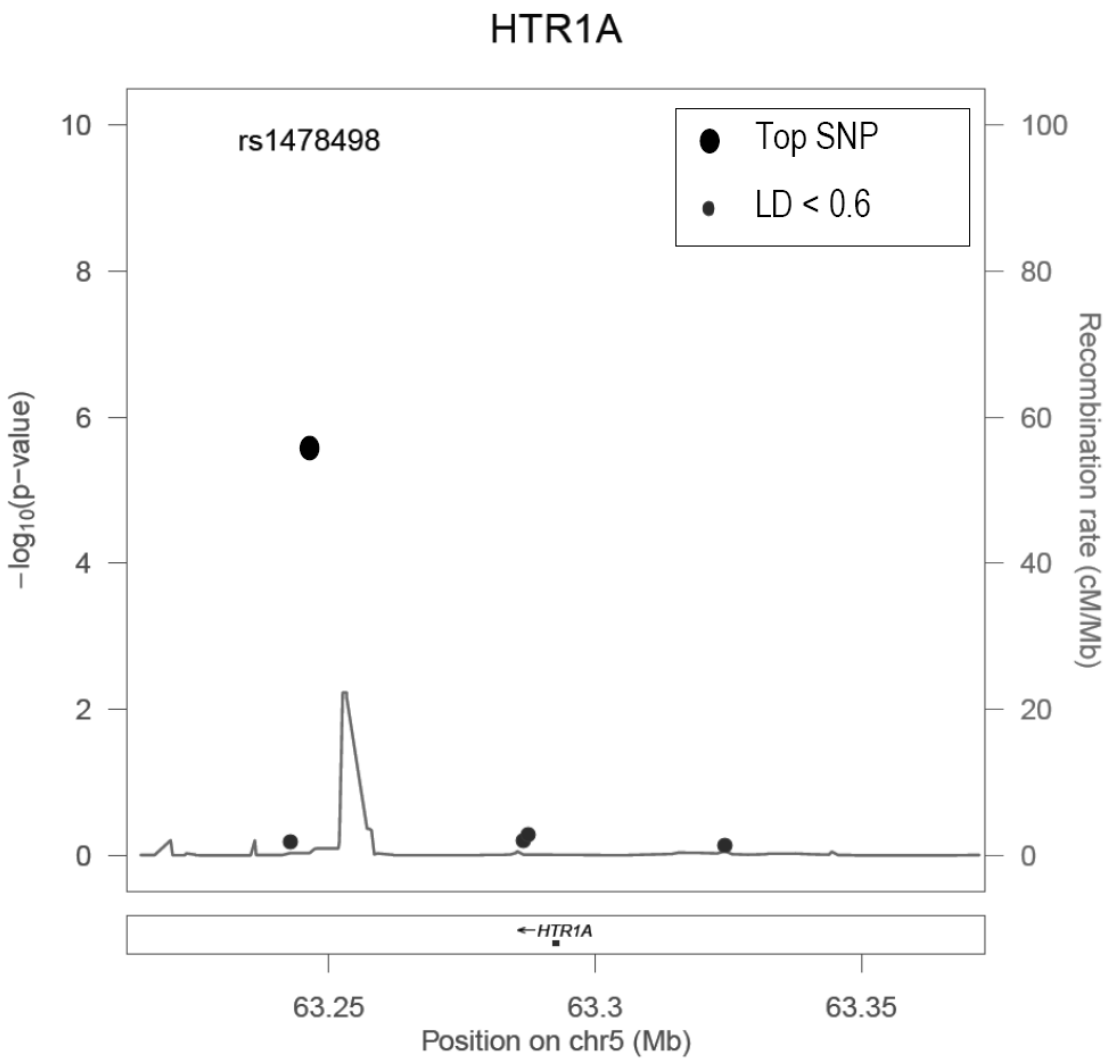

Figure 57: Plot of HTR1A SNPs - B
Only one SNP of the HTR1A gene (defined as ± 50kb) has a low p value, rs1478498, which was the 2nd most significant SNP overall (see Table 49). The other SNP are not in LD with the top SNP (LD < 0.6). The plot includes the complete LD region (LD ≥ 0.4) of the top SNP. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The plot was generated using LocusZoom (Pruim et al., 2010).

4.7. Results Body Dissatisfaction

4.7.1. Results genome-wide association SNP analysis - BD

The top SNP associated with Body Dissatisfaction (BD) scores is rs10485408 with a p value of 3.21E-06, this SNP is located on chromosome six within 50kb of the gene MAP3K7. General note: gene names are listed alphabetically in the

Appendix (see Table 61 to Table 65, page 244). None of the SNP p values pass a Bonferroni correction for multiple SNP testing, nor do they pass a Bonferroni threshold for suggestive association (0.5/283,744 = 1.76E-06). Figure 58 shows a plot of the SNP p values and the QQ-plot; the p values are generally normally distributed.

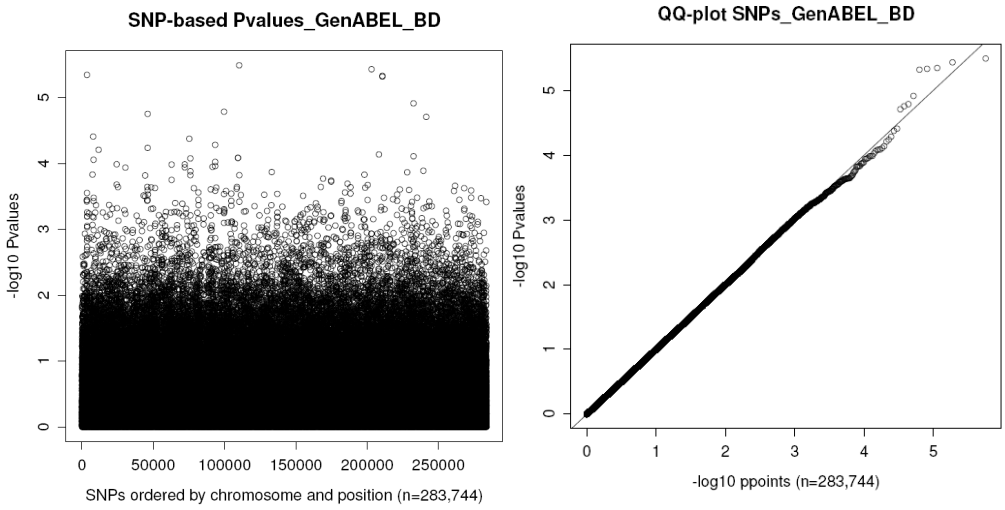

Figure 58: P value and QQ-plot – BD
The top SNP has a p value of 3.21E-06, which does not pass multiple SNP testing correction. The QQ-plot shows that the p values generally do not deviate from normality. Plots were generated using R software.

4.7.2. Results genome-wide association gene analysis - BD

The top gene for Body Dissatisfaction (BD) according to the VEGAS-Sum method is heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) with a p value of 1.30E-05 (see Table 50). It does not pass a Bonferroni correction for multiple gene testing, but it does pass a Bonferroni threshold for suggestive association (0.5/17,555 = 2.85E-05). HNRNPH1 ranked 31st according to the GATES method (see Table 50). According to VEGAS (UCSC Genome browser hg18 assembly) annotation there are nine SNPs in the gene, however according to GATES annotation (NCBI, also see paragraph 4.4.3 *Genome-wide gene analyses*, page 165) there are five SNPs in the gene. For pragmatic reasons (it is much more elusive to extract SNPs per gene ± 50kb using the UCSC Genome browser) NCBI annotation was used to plot the SNPs of the HNRNPH1. Out of the five SNPs

three have a p value below 0.0006, these three SNPs are not in strong LD (LD < 0.6, see Figure 59). The plot shows that there is another gene at this location, C5orf60. This gene was not part of the VEGAS gene set, but it ranked 28th out of 30,204 genes according to the GATES method (data not shown).

Results VEGAS method - BD

Rank	*Rank in GATES*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	31	HNRNPH1	5	9	1.30E-05	rs6875168	72
2	91	DNASE2B	1	8	2.16E-04	rs3121147	96
3	118	LEFTY2	1	12	3.54E-04	rs12743653	106
4	130	PYCR2	1	13	4.10E-04	rs12743653	106
5	***2***	***TFAP2E***	***1***	***4***	***4.35E-04***	rs7523017	3
6	79	TH1L	20	11	4.45E-04	rs6070696	80
7	#N/A	LOC441054	4	13	4.64E-04	-	-
8	***1***	***NCDN***	***1***	***3***	***5.46E-04***	rs7523017	3
9	194	HSD17B12	11	21	5.46E-04	rs1518821	134
10	***8***	***RUFY1***	***5***	***15***	***5.46E-04***	rs6894268	7
11	41	PRR16	5	38	5.51E-04	rs716815	12
12	72	PARP16	15	12	6.20E-04	rs665287	101
13	147	PLA2G12A	4	11	7.51E-04	rs7439493	314
14	33	EIF2C3	1	9	7.65E-04	rs538638	154
15	493	IBSP	4	17	7.93E-04	rs10018094	415
16	***24***	***UFSP2***	***4***	***16***	***8.56E-04***	rs6818425	67
17	59	RXRB	6	21	8.73E-04	rs756441	70
18	80	CTSZ	20	12	9.08E-04	rs6070696	80
19	32	ARG2	14	13	9.17E-04	rs3742879	59
20	560	VTI1B	14	8	9.59E-04	rs12050393	1754
21	50	TUBB1	20	13	9.89E-04	rs6070697	62
22	150	SAPS1	19	15	1.00E-03	rs12611091	354
23	103	SFRP2	4	5	1.04E-03	rs2251997	329
24	193	CASP6	4	13	1.12E-03	rs7439493	314
25	21515	RDH11	14	8	1.34E-03	rs1007284	>65,000

Table 50: Results VEGAS method – Top 25 genes BD

HNRNPH1 is the top gene most strongly associated with BD scores, it does not pass a Bonferroni multiple testing correction but it does pass a threshold for suggestive association (0.5/17,555 = 2.85E-05). HNRNPH1 is located on chromosome five and has nine SNPs according to VEGAS (UCSC) annotation, its top SNP ranked 72nd of SNPs overall. Some genes share a top SNP (an example in grey). Three genes were also among the top 25 genes according to the GATES method (in ***bold***). One gene was not part of the GATES gene set (indicated by #N/A).

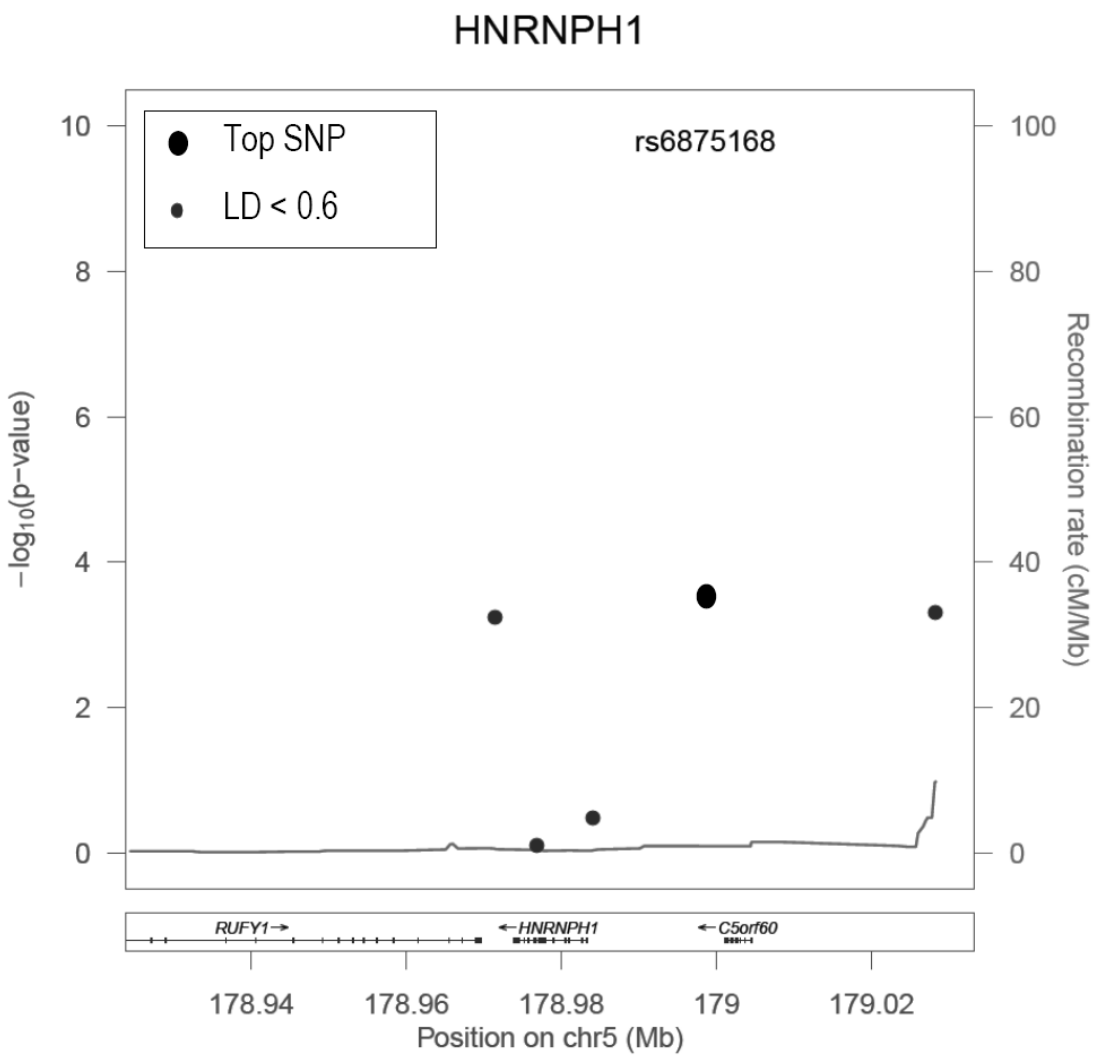

Figure 59: Plot of HNRNPH1 SNPs - BD
According to the GATES method (NCBI) five SNPs were annotated to the HNRNPH1 gene. Three SNPs have a p value below 0.0006. None of the SNPs are in strong LD (LD < 0.06). The top SNP, rs6875168, is indicated by the largest circle. There is another gene at this location, C5orf60. This gene was not part of the VEGAS gene set, but it ranked 28th out of 30,204 genes according to the GATES method (data not shown). The plot includes the complete LD region (LD ≥ 0.4) of the top SNP. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The plot was generated using LocusZoom (Pruim et al., 2010).

The top gene associated with BD scores resulting from the GATES method is neurochondrin (NCDN) with a p value of 8.98E-06 (see Table 51). The genes in the top 25 do not pass a Bonferroni correction for the number of genes tested, but four genes do pass the threshold for suggestive association (0.5/30,204 = 1.66E-05); NCDN, TFAP2E, KIAA0319L, and LOC100419802. Of these genes

transcription factor AP-2 epsilon (TFAP2E) was also among the top 25 genes according to the VEGAS method (rank 6, indicated in ***bold***, see Table 51). These four genes however all share the same top SNP, rs7523017, which was ranked third most significant SNP overall for BD (indicated in grey in Table 51). Figure 60 shows clearly that the gene p values are all a result of this SNP p value (rs7523017, p= 4.50E-06); there are several small genes in the area, which all rank high as a consequence of the single strongly associated SNP (see Figure 60); in this situation it is the SNP that deserves most attention, and to a lesser extent the genes that ranked high as a consequence of this single SNP p value. Note: LOC100419802 is a pseudogene; based on its sequence it is considered not to code for a functional protein (but it could nevertheless have a regulatory function (Wang and Chang, 2011)). LOC100419802 was not part of the gene set based on which the plot was generated (Pruim et al., 2010), hence it is not visible in Figure 60.

Results GATES method - BD

Rank	*Rank in VEGAS*	*Gene*	*Chr*	*nSNPs*	*Pvalue*	*Top SNP*	*Rank SNP*
1	11	NCDN	1	2	8.98E-06	rs7523017	3
2	***6***	***TFAP2E***	***1***	***2***	***8.98E-06***	rs7523017	3
3	71	KIAA0319L	1	5	1.34E-05	rs7523017	3
4	#N/A	LOC100419802	1	5	1.34E-05	rs7523017	3
5	#N/A	LOC100505569	12	17	4.87E-05	rs10745708	2
6	50	LCP1	13	22	6.19E-05	rs1230472	4
7	#N/A	SH2D7	15	9	8.80E-05	rs12593575	6
8	***13***	***RUFY1***	***5***	***13***	***1.45E-04***	rs6894268	7
9	#N/A	RLFP	4	4	1.51E-04	rs292909	11
10	#N/A	LOC100420260	1	7	2.90E-04	rs12061487	14
11	122	ADAM30	1	8	3.34E-04	rs12061487	14
12	361	NBPF7	1	9	3.38E-04	rs12061487	14
13	#N/A	LOC767850	1	9	3.38E-04	rs12061487	14
14	33	CIB2	15	6	3.96E-04	rs1542101	16
15	111	NOTCH2	1	9	4.00E-04	rs12061487	14
16	40	C1orf216	1	2	4.65E-04	rs7367713	68
17	53	CD248	11	4	4.83E-04	rs3741367	42
18	621	IBTK	6	13	4.84E-04	rs11752161	17
19	#N/A	LOC100421142	4	8	5.41E-04	rs9307270	23
20	2174	CNTNAP4	16	47	6.20E-04	rs8048863	9
21	#N/A	LOC645001	19	2	6.49E-04	rs1076976	86
22	350	LYPD4	19	2	6.49E-04	rs1076976	86
23	34	ANKRD37	4	5	6.83E-04	rs6818425	67
24	***22***	***UFSP2***	***4***	***5***	***6.83E-04***	rs6818425	67
25	54	CLSPN	1	4	6.86E-04	rs7367713	68

Table 51: Results GATES method – Top 25 genes BD

None of the genes pass a Bonferroni correction for the number of tested genes, but the top four genes do pass a Bonferroni threshold for suggestive association with BD. These four genes however all share the same top SNP, rs7523017, which ranked third of SNPs overall (also see Figure 60). Three genes were also among the top 25 genes according to the VEGAS method (in ***bold***).

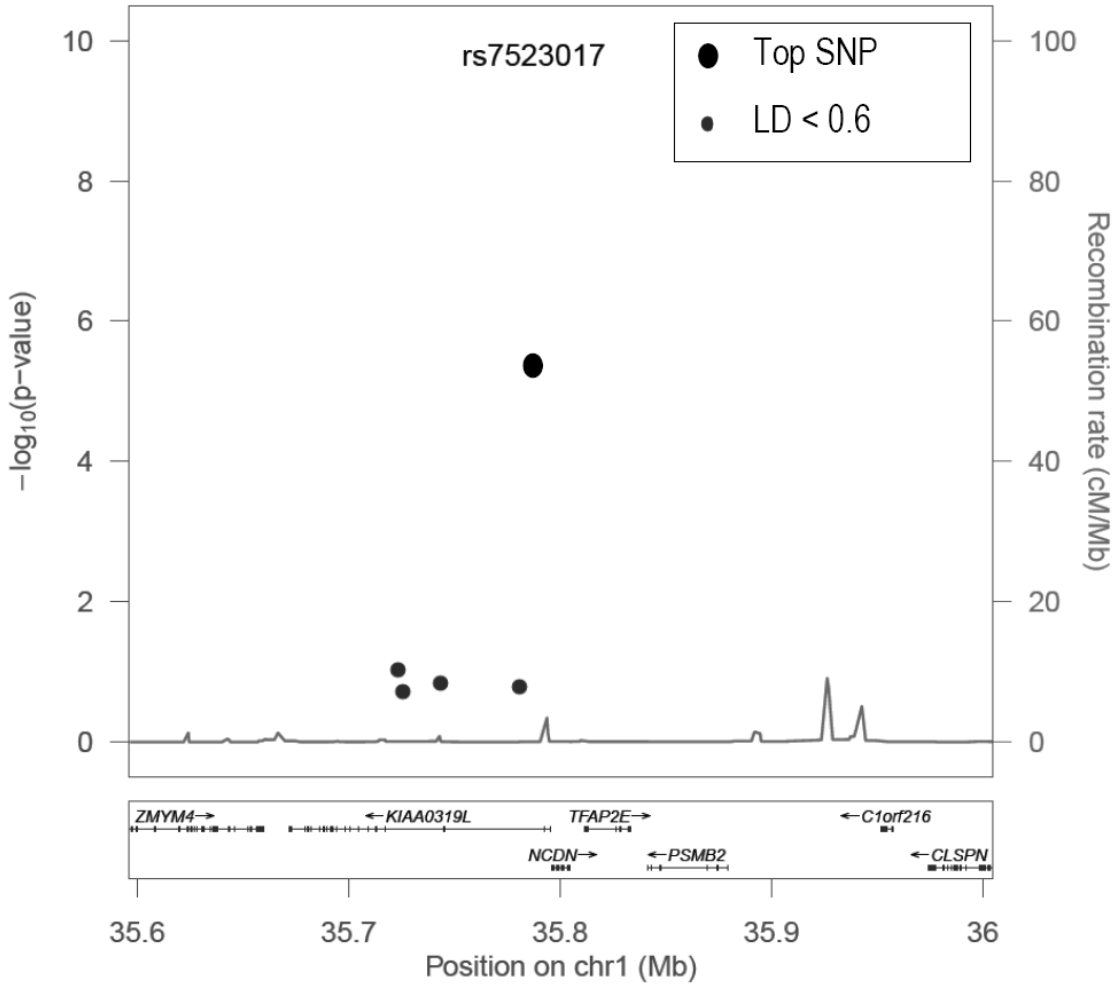

Figure 60: Plot of the SNPs from the top genes GATES – BD
This plot shows that the gene p values of the top genes for BD are a result of a single strongly associated SNP, rs7523017. The other four SNPs in the region are not in strong LD with the top SNP (LD < 0.6, indicated by the smaller circles), and they are not strongly associated to the phenotype either. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The plot was generated using LocusZoom (Pruim et al., 2010). An extended plot including the complete LD region ($LD \geq 0.4$) of the top SNP was presented in the Appendix, page 256.

The genes in this top 25 (see Table 51) stand out for having very few SNPs per gene compared to the other top 25s in this Chapter. The GATES method claims not to suffer from an inflation of the type I error due to gene size (Li et al., 2011),

which is important since by chance larger genes would be associated to any phenotype more often. However in this case there appears to be a suggestive bias towards smaller genes; the GATES method corrects the best p value of a gene for the number of independent SNPs in a gene, smaller genes are thus corrected less conservatively. The association was tested by a Spearman's rho test (a non-parametric statistic for a bivariate correlation on gene rank and the number of SNPs per gene); the number of SNPs is significantly associated with gene rank, however not smaller genes but larger genes tend to be ranked higher ($p<0.001$, correlation coefficient 0.046, n=30,204 genes). The impact of this association is however minor since the correlation coefficient is very small. This is illustrated in Figure 61 and Figure 62. Figure 61 shows that the mean rank increases with increasing number of SNPs per gene (indicated by bars), the distribution of genes (indicated by the line) shows though that there are very few large genes. Figure 62 only shows the smaller genes (100 SNPs or less per gene); the r-square of the number of SNPs per gene with the gene p value is very small (2.12E-05, see Figure 62), indicating that the deviation from zero is minimal. The association was tested by Spearman's rho rather than a linear regression since the p values may not be completely normally distributed, which would violate the assumption for linear regression. The p value is significant because the sample size is very large, but the correlation between the gene p value and the number of SNPs per gene is very small (0.046, see Figure 62), hence it is unlikely that the number of SNPs per gene have biased the results of the gene analyses for BD.

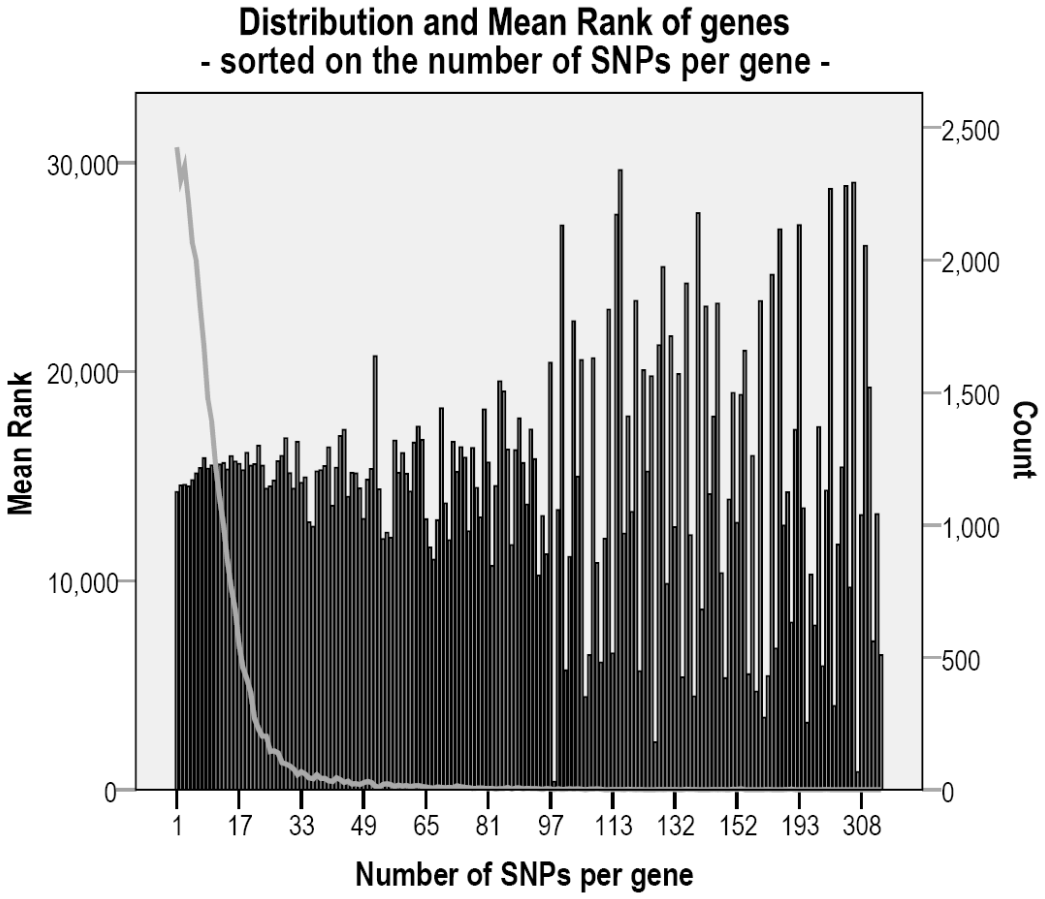

Figure 61: The mean rank of genes relates to the number of SNPs per gene
The left Y axis shows the mean rank of genes, and the right Y axis shows the number of genes – the genes are ordered on the number of SNPs per gene on the X axis. The rank of the gene increases with increasing number of SNPs per gene (Spearman's rho: $p<0.001$, correlation coefficient = 0.046, n= 30,204 genes). The impact of this association is however minor; the correlation coefficient is very small (also see Figure 62). There are very few large genes; most genes have less than 50 SNPs per gene.

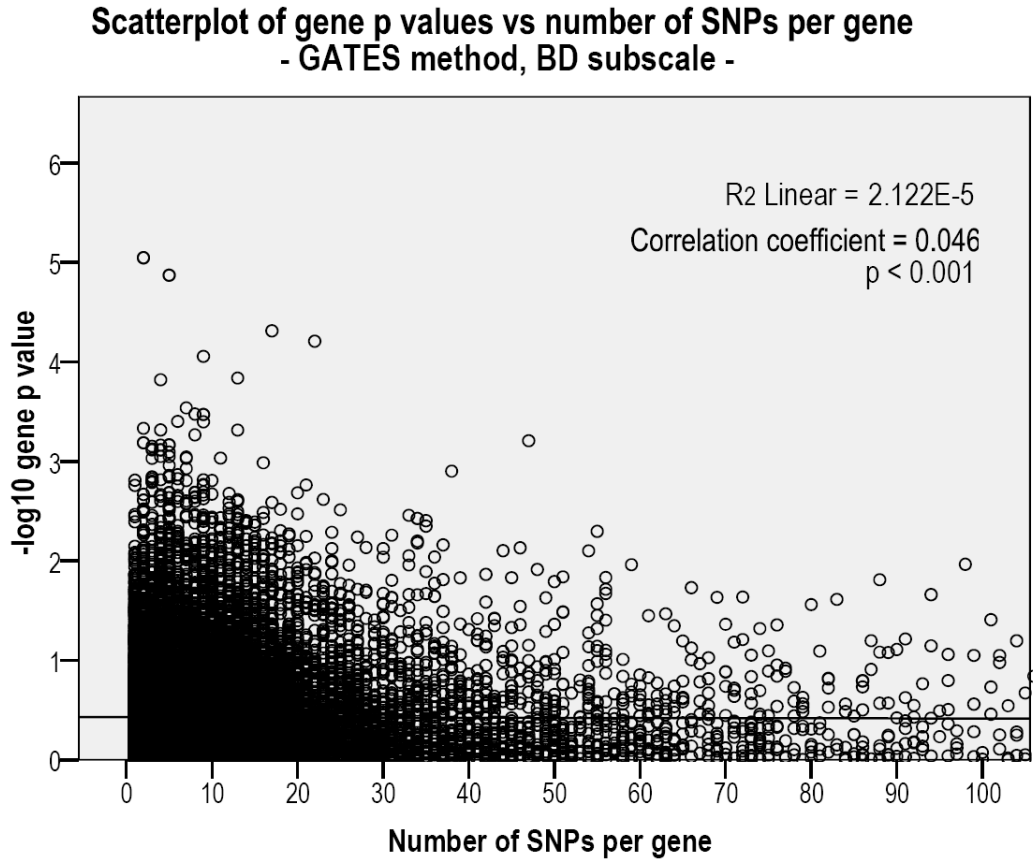

Figure 62: Scatterplot of gene p values versus the number of SNPs per gene
The gene p value is associated with the number of SNPs per gene, with lower p values associated with larger genes (Spearman's rho: correlation coefficient = 0.046, $p < 0.001$, n = 30,204 genes). The figure also shows the r-square value (2.12E-05, indicated by the line) in order to illustrate the minor impact of the association. Note: the association was not tested statistically by a linear regression but by Spearman's rho, since the p values may not be completely normally distributed. Only genes with 100 SNPs or less are shown in this figure.

4.8. Conclusion and discussion

No SNPs showed genome-wide significant association with drive for thinness (DT), bulimia (B), or body dissatisfaction (BD), as measured by the Eating Disorder Inventory (EDI), after correction for multiple SNP testing. Assuming there are indeed genetic variants underlying the variance in DT, B, and BD, scores (given the heritability estimates of the EDI (Rutherford et al., 1993,Keski-Rahkonen et al., 2005)(Boraska et al, submitted)) these results indicate that either the effect sizes were small, or that the causal common variants were not

well tagged (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45), or that the causal variants were rare variants (not at all tagged by current genotyping arrays). Results from studies on height and body mass index (Lango et al., 2010,Yang et al., 2011,Day and Loos, 2011), indicate that common genetic variants did underly phenotypic variance, but that large samples sizes were needed (50,000 to 200,000 individuals) in order to detect these because the effect sizes were very small. Though obviously challenging and time consuming, it would most definitely be possible to increase the sample size for the EDI questionnaire; it is a self report questionnaire which is easily administrered to the general population, and Podar and Allik demonstrated already that analyses of large cross-cultural samples of the EDI are feasible (n = 43,722) (Podar and Allik, 2009).

4.8.1. Secondary analyses

It is still a matter of debate whether secondary analyses can be conducted when the SNPs do not reach genome-wide significance ($p < 5.00E-08$ (Cichon et al., 2009)). The conservative side of the discussion is that the laws of probabilty should be honoured (Cichon et al., 2009,Kim et al., 2011); and most of the success has indeed resulted from increasing sample sizes rather than from novel analytical methods (Huang et al., 2011a,Lango et al., 2010,Yang et al., 2011,Day and Loos, 2011). From a biological perspective it is however not at all surprising that multiple different genetic risk factors could underly disease, and that the effects may be too small to pick up without aggregation into genes and pathways (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a), since genes are though to be the functional units of the human genome, and gene functions converge into pathways (also see the introduction of this chapter: paragraph 4.1, page 154). Perhaps this is even more the case in psychiatry, since neurodevelopmental processes are regulated by thousands of genes, with different functions at different timepoints in the development and functioning of the human brain (Rakic, 2009,State and Levitt, 2011). Moreover, because of the

importance of these basic brain processes there is much redundance in the biological processes involved; i.e. the system is very able to cope with minor 'flaws' (State and Levitt, 2011). The presumed genetic architecture of complex traits and disorders, consisting of multiple genetic risk variants each with a (very) small (cumulative) effect on risk (Visscher et al., 2011) (also see paragraph 1.3.4.3 *Undetected heritability and genetic architecture*, page 52), thus is biologically very plausible (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a).

The ideal proof of principle of secondary gene and pathway analyses would be to successfully analyse datasets with known genetic disease risk variants, which is possible because many datasets are open access these days (e.g. those of the Wellcome Trust Case-Control Consortium (WTCCC), http://www.wtccc.org.uk, (WTCCC, 2007)). Both Lehne *et al* (Lehne et al., 2011), and Huang *et al* (Huang et al., 2011a), took this approach to test multiple gene-based methods. None of the methods caused any false positive gene associations, but only a few of the known risk genes were picked up (seven out of 39 for Crohn's Disease (Lehne et al., 2011), five out of 27 for type 1 diabetes (Lehne et al., 2011), and six out of 38 for atherosclerosis risk (Huang et al., 2011a)). Lehne *et al* describe that some of the known risk genes consistently ranked low in all three gene-based methods they tested (Lehne et al., 2011); for some of these genes the truly associated SNPs were located more than 40kb away from the gene (Lehne *et al* extended gene regions by 40kb), or the associated SNP had not been genotyped in the open access data, or simply did not show any association (Lehne et al., 2011). Huang *et al* included the VEGAS method in their analyses, and they note that an important limitation of the approach is that the sum over all SNPs of a gene creates a bias to find causal variants in LD blocks represented by many SNPs, and that VEGAS would miss true disease risk variants located in LD blocks with fewer SNPs (Huang et al., 2011a). The most widely used approach is taking the most significant SNP p value per gene as the gene p value, however only when the size

of the gene or pathway (i.e. the number of SNPs per gene or pathway) is controlled for, because otherwise by chance large genes and pathways would be associated with the phenotype more often (Lehne et al., 2011,Li et al., 2011).

4.8.2. Associated genes did not pass correction for multiple testing

The results of this chapter indicate that none of the genes tested for association analyses with DT, B, and BD passed a threshold for multiple gene testing. It seems however almost more than coincidence that several previously with eating disorders or mental illness associated genes are among the top genes (including BDNF (Noble et al., 2011), GLP2R (Pinheiro et al., 2010), and HTR1A (Albert et al., 2011)). Any speculation about these genes would however be premature; larger sample sizes or independent replications would be necessary to verify these results. If however the results of the large scale genome-wide study of anorexia nervosa, currently conducted by the GCAN consortium, again point towards these genes (results will be published in 2012), then there may be enough evidence to decide to follow-up these genes, e.g. by sequencing them.

4.8.3. Appraisal of gene-based methods

The main aim of this chapter was to appraise the gene-based methods over SNP-based methods. The VEGAS-Sum methodology (Liu et al., 2010) fits the pressumed model of genetic architecture for complex traits and disorders (Visscher et al., 2011) much better than the GATES methodology (Li et al., 2011), because VEGAS-Sum combines the SNP p values per gene whereas GATES corrects the best p value of a gene for the number of SNPs in a gene (also see paragraph 4.4.3 *Genome-wide gene analyses*, page 165). VEGAS-Sum is indeed able to prioritise genes with multiple independent suggestive association signals; genes ranking high according to VEGAS-Sum would mostly not have been picked up if only the top SNPs associated with the DT, B, and BD scores had been considered for follow up (see Table 45, Table 48, and Table 50). This is in sharp contrast with the results following the GATES methodology; the top SNPs of all genes ranking high in GATES were among the top 70 SNPs overall (see Table 47,

Table 49, and Table 51). GATES gene-based methodology thus appears to add little to SNP-based association; it might be more straight-forward to simply follow up the genes of the top 100 SNPs, rather than performing gene-based association. Moreover, there is a slight bias towards smaller genes in the GATES methodology; smaller genes (with less SNPs) are corrected less conservatively than larger genes (see Figure 61 and Figure 62, page 189). Lehne *et al* also addressed this problem; they noted that small genes tended to be at the extremes of the p value distribution, i.e. the gene p value of a gene with one or two SNPs is dominated by these particular SNPs, in contrast to larger genes with more SNPs (Lehne et al., 2011). This bias is however only minor (see Figure 61 and Figure 62, page 189), more importantly to my opinion is that the GATES methology (only taking the most significant SNP of the gene into consideration) is a poor fit for the presumed genetic architecture of complex traits and disorders (Visscher et al., 2011).

4.8.4. Limitations

The limitations of the GATES methodology were most clear for the results of the pathway analyses. GATES-Simes pathway analyses methodology (Gui et al., 2011) is similar to the gene-based methodology (Li et al., 2011), in that the best gene of the pathway is corrected for the number of genes in the pathway. The pathway most significantly associated with DT was the Glucagon Type Ligand Receptors pathway (data presented in the Appendix, see Table 66, page 250). GLP2R is however the only gene of the pathway associated with DT with a p value below 0.05 (Table 66, page 250), moreover the top SNP of GLP2R was the second most significant SNP overall Table 47, page 176); it thus appears that a single SNP p value caused a whole pathway to be suggestively associated with the phenotype (note: the pathway p value did not pass a correction for multiple pathway testing), while there was no evidence for any of the other genes in the pathway to be even suggestively associated with the phenotype. Hence calculating gene and pathway p values by a correction of the best SNP appears to cause a risk of

overanalysing; it would be more accurate and appropriate to only state that the SNP rs7218549, located on chromosome 17, near the gene GLP2R, was suggestively associated with DT, in stead of stating that the gene GLP2R and the pathway Glucagon Type Ligand Receptors were suggestively associated with DT.

A major challenge in genome-wide association studies at any level, including SNP, gene, and pathway, is linkage disequilibrium (LD) (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a). SNPs and genes and pathways are not independent of each other (also see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, page 45). Moreover, as Huang *et al* point out, even when controlling for LD like in the VEGAS method (Liu et al., 2010) a bias can still result from the fact that regions in LD carrying many SNPs will be overrepresented in the results (Huang et al., 2011a). The results of this thesis indicate that the extended gene regions may exaggerate this problem; Figure 53 (page 175) shows that genes located closely together overlap almost completely when gene regions are extended, and Figure 71 (presented in the Appendix, page 253) shows that genes located closely together may not be in LD with each other, but when gene regions are extended there may be significant LD between genes. Lehne *et al* circumvent this problem by excluding genes from the major histocompatability complex (MHC) region (known to be in strong LD) from their gene-based analyses (indeed the genes from both Figure 53 and Figure 71 are located in this region) (Lehne et al., 2011), though the issue with LD may be more wide spread than just the MHC region.

4.8.5. Concluding remarks

As a final remark I wanted to emphasize that any output of an equation can only be as good as the input; i.e. when SNP p values are the input for gene and pathway association analyses, the results will only be as good as the SNP association with the phenotype. Genotyped SNPs in genome-wide association studies are merely 'tags' for a genomic region of common genetic variation (also

see paragraph 1.3.4.1 *Linkage disequilibrium, and multiple testing*, paragraph 45), and the genotyped SNPs are merely in LD with possible causal genetic risk variants. The very first genome-wide study conducted by Klein *et al* in 2005 is still a source of inspiration today; Klein and colleagues emphasized the quality of phenotyping, and provided compelling evidence for biological plausibility of the genome-wide significant association they found (Klein et al., 2005).

Conclusion and discussion

5. Conclusion and discussion

5.1. *Genetics of eating disorders*

Genetic studies of eating disorders (EDs), i.e. studies that aim to discover which genes are involved in the aetiology of EDs, have had limited success. Few if any, of these studies have identified (and replicated) robust genetic associations with EDs, and the results of this thesis (Chapter 2 and Chapter 4) are no exception to this rule. This 'failure' is not specific to EDs, and it has actually held important clues about the underlying genetic architecture of disease. Recent large-scale genetic studies of other complex (psychiatric) disorders and general traits such as human intelligence, height, and body weight, now confidently demonstrate that there are many genetic risk variants underlying complex disorders and traits, each either with a (very) small effect size, or (very) rare in the population (Cichon et al., 2009,Speliotes et al., 2010,State and Levitt, 2011,Davies et al., 2011,Lango et al., 2010). Power to detect genetic risk variants with such small effect sizes could be obtained by increasing samples sizes, and by focussing on disease related traits rather than diagnoses, which may have a more direct relationship with the underlying genetic risk factors.

5.2. *Quantitative traits of eating disorders*

Several quantitative traits, or endophenotypes, have been suggested for EDs (reviewed by Bulik *et al* (Bulik et al., 2007a), and by Treasure (Treasure, 2007)). Endophenotypes have been suggested to lead to more successful genetic analyses (Gottesman and Gould, 2003), though in reality they may be equally complex as diagnoses (Flint and Munafo, 2007,Walters and Owen, 2007). Drive for thinness (DT) and body dissatisfaction (BD) are among the most robust endophenotypes for EDs (Bulik et al., 2005,Bulik et al., 2007a,Stice and Shaw, 2002,Wilksch and Wade, 2009). Several studies show that DT and BD are

moderately heritable (Rutherford et al., 1993,Klump et al., 2000,Keski-Rahkonen et al., 2005,Boraska et al., submitted), and DT and BD have been included as behavioural covariates in genetic analyses of EDs before (Devlin et al., 2002,Root et al., 2011). When interpreting the endophenotype scores, it is important to take note of the context in which the (in this case) questionnaire was administered. The sample I analysed for Chapter 3 and Chapter 4 of this thesis was a general population sample, of older age and of higher BMI than a typical eating disorder study sample. Even though EDs are most common among adolescents, disordered eating and weight and shape concerns do also occur in women of older age (Lewis and Cachelin, 2001,Mangweth-Matzek et al., 2006,Bedford and Johnson, 2006,Slevec and Tiggemann, 2011).

The results of Chapter 3 of this thesis demonstrate that drive for thinness (DT), bulimia (B), and body dissatisfaction (BD) scores, as assessed by the Eating Disorder Inventory (EDI (Garner, 2004)) tend to be higher in individuals with a higher body mass index (BMI), which is in line with the literature (Packianathan et al., 2002,Mangweth-Matzek et al., 2006,Slevec and Tiggemann, 2011). Notably, highest adult lifetime BMI, rather than current BMI, was the most important predictor of DT and B scores. Current BMI, perhaps understandably, was however the best predictor for BD, though highest adult lifetime BMI still explained a significant proportion of unique variation in BD scores when current BMI was controlled for. This result may hold important implications for the assessment of disordered eating behaviour; highest adult lifetime BMI is easy to assess, and most individuals appear willing and able to report their highest adult lifetime BMI on self-report questionnaires (data presented in Chapter 3, page 119). Lifetime BMI history could be used as a proxy, or a risk factor, of disordered eating in the general population; e.g. to select individuals at risk for disordered eating in a classic two-stage study design as recommended by Hoek *et al* (Hoek and van Hoeken, 2003).

The effect sizes of the associations between DT, B, and BD, and BMI were considerable (the standardised correlation coefficients (beta) were 0.34, 0.32, and 0.53 for DT, B, and BD respectively). Individuals with low DT scores (below 13, EDI-3 scoring) have a mean BMI in the normal weight range (BMI between 20 and 25), and individuals with higher DT scores (13 and above) tend to have a BMI in the overweight range (BMI above 25). The relation between BD and BMI is most strong; individuals with low BD scores (below 35, EDI-3 scoring) are generally in the normal weight range, individuals with moderate BD scores (between 35 and 50) are in the overweight range (BMI between 25 and 30), and individuals with high BD scores (50 and above) tend to be obese (BMI above 30).

This highly significant relationship between DT, B, and BD scores and BMI does however not hold true for 5% of the sample (approximately 150 out of 3,000 individuals). These individuals present with high DT, B, and BD scores, without having, or ever having had, a high BMI. The percentage of individuals is approximately equal to the estimated prevalence of EDNOS in the general population (Hoek and van Hoeken, 2003,Wade et al., 2006,Machado et al., 2007,Keski-Rahkonen et al., 2007,Swanson et al., 2011), and could indicate that this group of outliers could consist of individuals at risk for EDs, with clinical EDs, or with a history of EDs. This finding again indicates that it is important to view disordered eating behaviour in the context of individual current BMI, and BMI history. Whether this subgroup of individuals with high DT, B, and BD scores, without ever having had a high BMI, truly represents possible disordered eating behaviour cases would have to be ascertained through (semi-)structured clinical interviews, but this is nevertheless a finding worthy of follow-up.

5.3. *Genome-wide gene analyses*

Genome-wide association (GWA) studies genotype single nucleotide polymorphisms (SNPs) in the genome. SNPs are however merely 'tags' of possible genetic risk variants for disease; genes are thought to be the functional

units of the human genome, and disease causing genetic variants should somehow influence gene function. Secondary analyses, e.g. genome-wide gene and genome-wide pathway analyses, are post-hoc to GWA SNP analyses. It is currently still a matter of debate whether secondary analyses can be conducted when the SNPs do not reach genome-wide significance ($p < 5.00E-08$ (Cichon et al., 2009)). From a biological perspective it would however not be surprising if multiple different genetic risk factors could underly the same disorder, and that the effects of the individual genetic risk factors may be too small to be detected without their aggregation into genes and pathways (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a). This may be particularly relevant for psychiatric disorders, since neurodevelopmental processes are regulated by thousands of genes (Rakic, 2009,State and Levitt, 2011).

Two different methods for secondary analyses of genome-wide gene associaton were tested in this thesis; the VEGAS-Sum method (Liu et al., 2010) which combines the SNP p values per gene, and the GATES method (Li et al., 2011), which corrects the best SNP of a gene for the number of SNPs in a gene. The VEGAS-Sum methodology fits the pressumed model of genetic architecture for complex traits and disorders better than the GATES methodology does; GATES is designed to detect genes with one or a few disease-susceptibility loci and relatively many neutral SNPs (Li et al., 2011), whereas VEGAS-Sum is geared for detecting genes with multiple disease-susceptibility loci (Liu et al., 2010,Li et al., 2011), exactly the type of genetic variations expected to underly complex disorders such as eating disorders (Visscher et al., 2011). The VEGAS-Sum method is indeed able to prioritise genes with multiple independent suggestive association signals; genes detected by the VEGAS-Sum method would mostly not have been picked up using only primary GWA SNP analyses. This is in sharp contrast with the genes detected by the GATES method; the top SNPs of these genes were all among the top 70 SNPs overall, indicating that these genes would

have been identified if simply the top 100 SNPs was followed up without doing any secondary analyses.

Linkage disequilibrium (LD) represents a major challenge for genome-wide association studies at any level, including SNP, gene, and pathway (Cantor et al., 2010,Lehne et al., 2011,Huang et al., 2011a). Huang *et al* point out for example, that gene-based association methods like the VEGAS-Sum method, which take the sum over all SNPs of a gene, can create a bias towards finding causal variants in LD blocks carrying many SNPs, and miss causal variants in LD blocks represented by fewer SNPs (Huang et al., 2011a). The results of this thesis indicate that extended gene regions may further exaggerate this problem; genes located closely together may not be in LD with each other, but when gene regions are extended there can be significant LD between genes. Lehne *et al* circumvent this problem by excluding genes from the major histocompatability complex (MHC) region (known to be in strong LD) from their gene-based analyses (Lehne et al., 2011). Indeed the most problematic LD regions found in this thesis (Figure 53 and Figure 71) were located in this region, but the issue with LD may be more wide spread than just the MHC region.

No SNPs or genes were found to be significantly associated with the Eating Disorder Inventory (EDI) (Garner, 2004) risk scales drive for thinness (DT), bulimia (B), or body dissatisfaction (BD) after correction for multiple testing. This is in line with other complex traits, such as human intelligence (Davies et al., 2011), height (Lango et al., 2010), and body mass index (Day and Loos, 2011); very much larger samples sizes were required to detect any genetic risk variants underlying these traits (in the region of tens of thousands of samples, rather than the approximately two thousand samples analysed in this thesis). Increasing the sample size is however not a guarantee for success. The EDI scales were chosen as candidate endophenotypes for eating disorders; i.e. simpler phenotypes than

the complex diagnoses, meant to lead to more straight forward, and more successful genetic analyses (Gottesman and Gould, 2003). Whether the EDI scales are good endophenotypes for eating disorders, and whether they truly associate with eating disorder causing genetic variants (Flint and Munafo, 2007,Walters and Owen, 2007) however remains to be ascertained. The results of this thesis are – despite the lack of significance – very encouraging, because among the top genes were several previously implicated in the aetiology of eating disorders (including BDNF (Noble et al., 2011), GLP2R (Pinheiro et al., 2010), and HTR1A (Albert et al., 2011)). In 2012 the results of the first large scale genome-wide study of anorexia nervosa by the GCAN consortium are expected to be published. If these genes are again among the top results, there would, to my opinion, be enough suggestive evidence to decide to follow them up, e.g. through sequencing studies.

5.4. *Future directions*

The complex clinical presentation of eating disorders, the presumed underlying molecular biology, and the statistical analyses methods to test causal relationships, are all challenging fields of science on their own, let alone when one tries to combine them in the genetic studies of eating disorders. Multidisciplinary collaborations between clinicians, molecular biologists, and statisticians are thus essential to make any progress in this field. Collaborations will also be needed because sample sizes will have to increase; it has become apparent that genetic risk variants underlying complex disorders and traits have (very) small effect sizes which can only be detected using very large samples (tens of thousands of cases). I have to agree with Kim *et al* that underpowered studies are not worth undertaking anymore (Kim et al., 2011), time and money would be better spent on recruiting more cases and collecting more data. Indeed promising progress is being made by researchers from consortia such as the Japanese Genetic Research Group for Eating Disorders (JGRED), the Price

Foundation Genetic Studies of Eating Disorders, and the Genetic Consortium for Anorexia Nervosa (GCAN).

The overlap of symptoms between eating disorder categories, the instability of eating disorder diagnoses over time, and the disproportionate number of patients with a diagnosis of eating disorder not otherwise specified (EDNOS) pose further challenges for the study of genetics of eating disorders. It is encouraging however that several genetic risk variants have now been identified for body mass index (BMI). BMI is not a stable phenotype either; it can fluctuate significantly over time, and its aetiology is arguably as multifactorial as that of eating disorders. Again the key word for success has been large sample sizes; a discovery sample of more than 100,000 individuals followed by a replication in more than 100,000 individuals has lead to the robust association of more than 50 genetic risk variants for BMI (Speliotes et al., 2010). Such sample sizes may simply not be feasible for eating disorders. Secondary analyses, such as gene and pathway association analyses may be able to identify genetic risk variants in smaller sample sizes. From a biological perspective it would not be surprising if multiple different genetic risk factors could underly the same disorder, hence studying them through their aggregation into genes and pathways could improve the chances of detecting the underlying mechanism of disease (Cantor et al., 2010). Currently these secondary analyses are still in their infancy, and proof of principle should ideally be obtained by successfully analysing datasets with known genetic disease risk variants (Lehne et al., 2011,Huang et al., 2011a). Given the comorbidity of eating disorders with other psychiatric disorders (Jacobi et al., 2004b,Hudson et al., 2007,Swanson et al., 2011,Dalle, 2011,Treasure et al., 2010), it may be worth combining samples of different psychiatric disorders. This may introduce heterogeneity, but larger sample sizes do have more power to detect genetic risk variants with small effect sizes.

Endophenotypes of complex disorders are an appealing concept, and they may lead to more successful genetic analyses (Gottesman and Gould, 2003), though success is not guaranteed (Flint and Munafo, 2007,Walters and Owen, 2007). The results of this thesis are encouraging though, among the top genes associated with drive for thinness, bulimia, and body dissatisfaction in a general population sample were several previously implicated in the aetiology of eating disorders. The results did not pass a correction for multiple testing, but compared to other studies of complex traits the sample I analysed was relatively small. It would be feasible to increase the sample size for quantitative trait measurements such as the Eating Disorders Inventory (EDI) (Garner, 2004), or other candidate quantitative traits for eating disorders such as for example, the Eating Disorder Diagnostic Scale (EDDS) (Stice et al., 2000), or measures of Weight Concerns (Killen et al., 1994). When using these measures it is important to take note of their observed dimensionality (Garcia-Grau et al., 2010). For example, future studies may want to run genetic analyses on the observed rather than the suggested factor structures, e.g. by combining the drive for thinness and body dissatisfaction items of the EDI, since analyses by Garcia-Grau *et al* demonstrate that they load onto the same factor (Garcia-Grau et al., 2010). One should also take note of the correlates of the candidate endophenotypes; e.g. the EDI scores are significantly associated with BMI. Whether higher EDI scores truly represent more disordered eating behaviour when accompanied by higher BMIs remains to be ascertained, it will be important to study disordered eating in the context of current BMI and BMI history. If higher EDI scores truly represent more disordered eating then it will also be important to include older age groups in research, since EDI scores were very equal between women of different age groups.

Apart from genetic variants in gene regions there are many more factors which could interfere with protein functionality. Very interesting to my opinion are epigenetic mechanisms (Campbell et al., 2011) and microRNAs (Huang et al.,

2011b). Both are important in the fine tuning of gene function, and it is plausible that they are involved in the aetiology of eating disorders. However, before they could be implicated in the aetiology of eating disorders it is very important that the clinical presentation is well captured by precise phenotyping. Hence, for the foreseeable future I think all effort should be focussed on obtaining extremely well documented very large cohorts, because progress in a scientific field so challenging can only result from collaborative multi-national multi-disciplinary labour.

5.5. Concluding remarks

What if eating disorders really have a genetic basis, would that mean there is nothing we can do about it? That they are inevitable? Unsolvable? No, most certainly not. Day and Loos discuss the 50 genetic risk variants that have now robustly been associated with obesity; the effect of body mass index-increasing genetic variants is however reduced by physical activity (Day and Loos, 2011). A genetic basis does imply though, that for some individuals it is truly more difficult to manage their eating behaviour than it is for others. This is not really novel, for example everybody knew already that it is more difficult for some people to deliver a speech than it is for other people (genetic factors contribute to the variability in cortisol changes in response to the Trier Social Stress Test (Kirschbaum et al., 1993)). The presumed genetic architecture of eating disorders also indicates that genetics would not be a good screening tool to identify people at risk for an eating disorder in the general population, as it is not for obesity (Day and Loos, 2011); it would be far more easy to buy a weighing scale and a measuring tape. The same holds true for anorexia nervosa. I have nevertheless been arguing for about 200 pages that genetics of eating behaviour is useful. And the answer to the unasked question following the previous statement is 'knowledge'. Though perhaps appropriate for a degree in philosophy, I realise this may sound a bit daft. What I mean is that, if we understand better why some individuals fall ill, we can help them better. If for example the studies on the

gene FTO, which was discovered to be associated with body mass index (Frayling et al., 2007), lead to a better understanding of why it is so difficult for some individuals to balance their energy intake and expenditure on a day to day basis, then maybe we can finally get rid of all the “crash-diet” and “weigh-less-in-3-minutes” books. Similarly, if it turns out that glucagon signalling is truly associated with eating disorders (suggestive evidence from (Pinheiro et al., 2010) and from Chapter 4 of this thesis), e.g. through impaired hunger signalling, then a simple drug may be able to pull a whole group out of a danger zone. Glucagon drugs will most certainly not be the answer to eating disorders; if there is one thing this thesis underscores then it is the complexity and multifactor”reality“ of eating disorders. Yet one new insight into the aetiology of eating disorders could be lifechanging for one individual.

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Appendix 1: HWE and HapMap frequency details for Chapter 2

*	SNP rs#	HWE p value	HapMap CEU homozygote	Control sample homozygote	Difference	HapMap CEU heterozygote	Control sample heterozygote	Difference
x	rs987778	0.51	0.000	0.000	0.000	0.107	0.049	0.058
	rs2917928	0.24	0.433	0.432	0.001	0.450	0.434	0.016
x	rs4680	0.57	0.292	0.293	0.001	0.460	0.486	0.026
	rs10444117	0.77	0.858	0.860	0.002	0.142	0.136	0.006
	rs363221	0.80	0.881	0.884	0.003	0.119	0.113	0.006
x	rs1801153	0.29	0.619	0.625	0.006	0.319	0.340	0.021
	rs7863731	0.63	0.367	0.361	0.006	0.517	0.472	0.045
x	rs2383378	0.57	0.381	0.374	0.007	0.451	0.484	0.033
	rs6355	0.59	0.950	0.959	0.009	0.050	0.041	0.009
	rs17210001	***0.0007***	0.876	0.886	0.010	0.124	0.095	0.029
	rs3808955	0.93	0.018	0.007	0.011	0.177	0.161	0.016
	rs2020933	0.60	0.900	0.889	0.011	0.100	0.107	0.007
	rs12728678	0.85	0.627	0.645	0.018	0.322	0.315	0.007
	rs11564771	0.41	0.850	0.871	0.021	0.142	0.122	0.020
	rs709611	0.56	0.116	0.094	0.022	0.429	0.409	0.020
x	rs7836907	0.22	0.788	0.765	0.023	0.204	0.226	0.022
x	rs12831013	0.32	0.900	0.925	0.025	0.100	0.075	0.025
	rs363251	0.68	0.115	0.151	0.036	0.469	0.465	0.004
	rs11264262	0.26	0.770	0.807	0.037	0.212	0.179	0.033
	rs1390939	***0.07***	0.159	0.209	0.050	0.549	0.457	0.092
x	rs1718312	0.76	0.460	0.403	0.057	0.416	0.459	0.043
	rs1126758	0.36	0.212	0.154	0.058	0.416	0.498	0.082
	rs4633	0.85	0.288	0.223	0.065	0.459	0.495	0.036
x	***rs7820517***	***0.05***	0.763	0.674	0.089	0.220	0.307	0.087
x	rs2173114	0.22	0.167	0.262	0.095	0.567	0.474	0.093
	rs1055663	0.57	-	0.227	-	** 0.496	0.510	0.014

Table 52: HWE and HapMap frequencys - all SNPs Chapter 2

SNPs are sorted on the difference between the HapMap and sample reference homozygote genotype frequencies. Control sample consisted of London and Vienna control samples (n= 674). All SNPs genotype frequencies are ± 10% of the HapMap frequencies (maximum difference is 0.095). * x indicate published SNPs. ** HapMap genotype frequencies were not available, but the average heterozygosity of this SNP was 0.496. One SNP violates HWE significantly (p= 0.0007, rs17210001, in ***bold***), two SNPs are suggestive of HWE violation (in ***bold***). Rs4633 was excluded from analyses (see paragraph 2.5, page 77), it is shown in grey.

Appendix 2: Additional data for Chapter 2

Results case control analyses - genotypic and allelic association

*	SNP		Allele 1	Heterozygote	Allele 2	n	Missing	p genotype	p allele
	rs10444117	Case	310 (86%)	51 (14%)	0 (0%)	361	2%	0.27	0.89
		Control	577 (86%)	91 (14%)	3 (0%)	671	0%		
	rs1055663	***Case***	***95 (26%)***	***195 (54%)***	***74 (20%)***	***364***	***1%***	***0.08***	***0.04***
		Control	***152 (23%)***	***342 (51%)***	***176 (26%)***	***670***	***1%***		
	rs11264262	Case	289 (79%)	71 (19%)	6 (2%)	366	0%	0.81	0.52
		Control	542 (81%)	120 (18%)	10 (1%)	672	0%		
	rs1126758	Case	58 (16%)	188 (51%)	121 (33%)	367	0%	0.83	0.60
		Control	103 (15%)	334 (50%)	234 (35%)	671	0%		
	rs11564771	Case	256 (89%)	33 (11%)	0 (0%)	289	21%	0.19	0.38
		Control	512 (87%)	72 (12%)	4 (1%)	588	13%		
	rs12728678	Case	225 (62%)	120 (33%)	16 (4%)	361	2%	0.79	0.49
		Control	432 (64%)	211 (31%)	27 (4%)	670	1%		
x	rs12831013	Case	329 (92%)	29 (8%)	1 (0%)	359	2%	0.33	0.51
		Control	621 (93%)	50 (7%)	0 (0%)	671	0%		
	rs1390939	Case	115 (32%)	180 (50%)	64 (18%)	359	2%	0.33	0.73
		Control	220 (33%)	300 (46%)	137 (21%)	657	3%		
x	rs1718312	Case	34 (10%)	168 (49%)	143 (41%)	345	6%	0.19	0.27
		Control	89 (14%)	297 (46%)	261 (40%)	647	4%		
	rs17210001	Case	112 (85%)	18 (14%)	1 (1%)	131	64%	0.34	0.63
		Control	186 (89%)	20 (10%)	4 (2%)	210	69%		
x	***rs1801153***	***Case***	***185 (68%)***	***73 (27%)***	***14 (5%)***	***272***	***26%***	***0.08***	***0.35***
		Control	***358 (62%)***	***195 (34%)***	***20 (3%)***	***573***	***15%***		
	rs2020933	Case	326 (89%)	38 (10%)	1 (0%)	365	1%	0.90	0.77
		Control	598 (89%)	72 (11%)	3 (0%)	673	0%		
x	*rs2173114*	*Case*	*36 (21%)*	*91 (53%)*	*46 (27%)*	*173*	*53%*	*0.31*	*0.36*
		Control	*144 (26%)*	*260 (47%)*	*145 (26%)*	*549*	*19%*		

Table 53: Results case control analyses - all SNPs Chapter 2 (1)

* x indicate published SNPs. Some SNPs are suggestively associated with AN (in ***bold***), however taking multiple testing into account none of the SNPs are associated with AN.

Results case control analyses - continued

*	SNP		Allele 1	Heterozygote	Allele 2	n	Missing	p genotype	p allele
x	rs2383378	Case	131 (36%)	174 (48%)	56 (16%)	361	2%	0.84	0.59
		Control	250 (37%)	323 (48%)	95 (14%)	668	1%		
	rs2917928	Case	40 (13%)	123 (40%)	146 (47%)	309	16%	0.50	0.34
		Control	80 (13%)	259 (43%)	258 (43%)	597	11%		
	rs363221	Case	324 (89%)	40 (11%)	0 (0%)	364	1%	0.42	0.68
		Control	596 (88%)	76 (11%)	2 (0%)	674	0%		
	rs363251	Case	39 (12%)	153 (47%)	137 (42%)	329	10%	0.33	0.16
		Control	95 (15%)	293 (47%)	242 (38%)	630	7%		
	rs3808955	***Case***	***0 (0%)***	***71 (19%)***	***294 (81%)***	***365***	***1%***	***0.05***	***0.49***
		Control	***5 (1%)***	***108 (16%)***	***557 (83%)***	***670***	***1%***		
	rs4633	Case	80 (22%)	195 (53%)	92 (25%)	367	0%	0.47	0.57
		Control	148 (22%)	328 (49%)	187 (28%)	663	2%		
x	rs4680	Case	89 (26%)	176 (51%)	82 (24%)	347	5%	0.47	0.27
		Control	191 (29%)	317 (49%)	144 (22%)	652	3%		
	rs6355	Case	357 (98%)	8 (2%)	0 (0%)	365	1%	0.10	0.10
		Control	634 (96%)	27 (4%)	0 (0%)	661	2%		
	rs709611	***Case***	***17 (5%)***	***154 (42%)***	***193 (53%)***	***364***	***1%***	***0.02***	***0.05***
		Control	***63 (9%)***	***275 (41%)***	***334 (50%)***	***672***	***0%***		
x	rs7820517	Case	255 (69%)	106 (29%)	6 (2%)	367	0%	0.76	0.48
		Control	454 (67%)	207 (31%)	13 (2%)	674	0%		
x	rs7836907	Case	260 (77%)	72 (21%)	5 (1%)	337	8%	0.73	0.95
		Control	471 (76%)	139 (23%)	6 (1%)	616	9%		
	rs7863731	Case	134 (37%)	183 (50%)	48 (13%)	365	1%	0.30	0.35
		Control	240 (36%)	314 (47%)	111 (17%)	665	1%		
x	rs987778	Case	340 (95%)	18 (5%)	1 (0%)	359	2%	0.35	0.67
		Control	636 (95%)	33 (5%)	0 (0%)	669	1%		

Table 54: Results case control analyses - all SNPs Chapter 2 (2)
* x indicate published SNPs. Some SNPs are suggestively associated with AN (in ***bold***), however taking multiple testing into account none of the SNPs are associated with AN. Rs4633 was excluded from analyses, it is shown in grey.

Results within-case analyses - genotypic and allelic association - lowest BMI

*	SNP		Allele 1	Heterozygote	Allele 2	n	Missing	p genotype	p allele
	rs10444117	Case	429 (87%)	62 (13%)	0 (0%)	491	2%	0.78	0.79
	rs1055663	*Case*	*128 (26%)*	*270 (55%)*	*102 (21%)*	*500*	*1%*	*0.72*	*0.59*
	rs11264262	Case	388 (79%)	98 (20%)	14 (3%)	500	1%	0.25	0.78
	rs1126758	Case	74 (15%)	247 (50%)	180 (37%)	501	1%	0.75	0.47
	rs11564771	***Case***	***308 (63%)***	***52 (11%)***	***0 (0%)***	***360***	***22%***	***0.01***	***0.02***
	rs12728678	Case	324 (66%)	152 (31%)	20 (4%)	496	1%	0.72	0.84
x	rs12831013	Case	445 (91%)	41 (8%)	1 (0%)	487	3%	0.12	0.69
	rs1390939	Case	155 (32%)	234 (48%)	88 (18%)	477	4%	0.25	0.23
x	***rs1718312***	***Case***	***62 (13%)***	***218 (44%)***	***186 (38%)***	***466***	***6%***	***0.02***	***0.10***
	rs17210001	Case	113 (23%)	16 (3%)	1 (0%)	130	58%	0.69	0.42
x	*rs1801153*	*Case*	*220 (45%)*	*101 (21%)*	*17 (3%)*	*338*	*26%*	*0.75*	*0.84*
	rs2020933	Case	436 (89%)	51 (10%)	1 (0%)	488	3%	0.78	0.66
x	rs2173114	Case	39 (8%)	91 (19%)	44 (9%)	174	51%	0.38	0.43
x	rs2383378	Case	181 (37%)	240 (49%)	74 (15%)	495	2%	0.92	0.76
	rs2917928	Case	53 (11%)	167 (34%)	179 (36%)	399	16%	0.28	0.12
	rs363221	Case	439 (89%)	59 (12%)	1 (0%)	499	1%	0.37	0.49
	rs363251	***Case***	***40 (8%)***	***216 (44%)***	***181 (37%)***	***437***	***11%***	***0.02***	***0.21***
	rs3808955	*Case*	*2 (0%)*	*90 (18%)*	*408 (83%)*	*500*	*1%*	*0.63*	*0.44*
	rs4633	Case	101 (21%)	268 (55%)	133 (27%)	502	1%	0.38	0.19
x	rs4680	Case	132 (27%)	245 (50%)	102 (21%)	479	4%	0.42	0.20
	rs6355	***Case***	***485 (99%)***	***15 (3%)***	***0 (0%)***	***500***	***1%***	***0.03***	***0.03***
	rs709611	*Case*	*23 (5%)*	*212 (43%)*	*265 (54%)*	*500*	*1%*	*0.53*	*0.59*
x	rs7820517	Case	347 (71%)	141 (29%)	13 (3%)	501	1%	0.63	0.38
x	rs7836907	Case	336 (68%)	101 (21%)	5 (1%)	442	10%	0.11	0.14
	rs7863731	Case	175 (36%)	257 (52%)	65 (13%)	497	1%	0.53	0.30
x	rs987778	Case	473 (96%)	22 (4%)	1 (0%)	496	1%	0.44	0.46

Table 55: Results within-case analyses - all SNPs Chapter 2 - lowest BMI
* x indicate published SNPs. Some SNPs are suggestively associated with AN (in ***bold***), however taking multiple testing into account none of the SNPs are associated with AN. Lowest p value is 0.01 which does not pass the Bonferroni correction threshold for 26 SNPs: $\alpha = 0.05 / 26 \text{ SNPs} = 0.002$). Note: Percentage Missing refers to missing genotypes, lowest BMI was missing for 22% of the cases. Rs4633 was excluded from analyses, it is shown in grey.

Results within-case analyses - genotypic and allelic association - highest BMI

*	SNP		Allele 1	Heterozygote	Allele 2	n	Missing	p genotype	p allele
	rs10444117	***Case***	***378 (87%)***	***55 (13%)***	***0 (0%)***	***491***	***11%***	***0.07***	***0.13***
	rs1055663	Case	117 (27%)	239 (55%)	86 (20%)	500	10%	0.29	0.22
	rs11264262	*Case*	343 (79%)	86 (20%)	13 (3%)	500	10%	1.00	0.58
	rs1126758	*Case*	65 (15%)	217 (50%)	160 (37%)	501	10%	0.61	0.31
	rs11564771	***Case***	***267 (62%)***	***49 (11%)***	***0 (0%)***	***360***	***29%***	***0.08***	***0.60***
	rs12728678	*Case*	284 (66%)	137 (32%)	17 (4%)	496	10%	0.30	0.30
x	rs12831013	*Case*	387 (89%)	42 (10%)	1 (0%)	487	12%	0.46	0.93
	rs1390939	*Case*	137 (32%)	206 (48%)	77 (18%)	477	13%	0.69	0.79
x	rs1718312	Case	58 (13%)	189 (44%)	162 (37%)	466	15%	0.91	0.37
	rs17210001	*Case*	101 (23%)	14 (3%)	1 (0%)	130	60%	0.71	1.00
x	rs1801153	Case	193 (45%)	91 (21%)	14 (3%)	338	32%	0.12	0.80
	rs2020933	***Case***	***382 (88%)***	***46 (11%)***	***1 (0%)***	***488***	***12%***	***0.01***	***0.47***
x	rs2173114	*Case*	37 (9%)	76 (18%)	41 (9%)	174	54%	0.39	1.00
x	rs2383378	*Case*	151 (35%)	221 (51%)	65 (15%)	495	11%	0.86	0.84
	rs2917928	*Case*	42 (10%)	149 (34%)	160 (37%)	399	24%	0.68	0.97
	rs363221	*Case*	386 (89%)	54 (12%)	1 (0%)	499	10%	0.65	0.51
	rs363251	***Case***	***31 (7%)***	***194 (45%)***	***156 (36%)***	***437***	***19%***	***0.08***	***0.92***
	rs3808955	*Case*	*2 (0%)*	*81 (19%)*	*358 (83%)*	*500*	*10%*	*0.95*	*0.12*
	rs4633	*Case*	83 (19%)	235 (54%)	125 (29%)	502	10%	0.79	0.99
x	rs4680	*Case*	124 (29%)	216 (50%)	83 (19%)	479	13%	0.57	0.98
	rs6355	Case	428 (99%)	14 (3%)	0 (0%)	500	10%	0.89	0.38
	rs709611	Case	20 (5%)	186 (43%)	235 (54%)	500	10%	0.23	0.87
x	rs7820517	*Case*	308 (71%)	122 (28%)	13 (3%)	501	10%	0.57	0.92
x	rs7836907	*Case*	293 (68%)	89 (21%)	3 (1%)	442	19%	0.41	0.81
	rs7863731	*Case*	156 (36%)	228 (53%)	54 (12%)	497	10%	0.72	0.21
x	rs987778	*Case*	418 (97%)	19 (4%)	1 (0%)	496	10%	0.42	0.34

Table 56: Results within-case analyses - all SNPs Chapter 2 - highest BMI
* x indicate published SNPs. Some SNPs are suggestively associated with AN (in ***bold***), however taking multiple testing into account none of the SNPs are associated with AN. Lowest p value is 0.01 which does not pass the Bonferroni correction threshold for 26 SNPs: $\alpha = 0.05 / 26 \text{ SNPs} = 0.002$). Note: Percentage Missing refers to missing genotypes, highest BMI was missing for 31% of the cases. Rs4633 was excluded from analyses, it is shown in grey.

Haplotype	*Case (n)*	*Frequency*	*p value*
C-G-G	251	37%	0.17
T-G-G	290	43%	0.13
T-A-G	104	15%	0.78
T-A-C	31	5%	0.92

Test of overall association: chisq= 2.6, df= 3, p value= 0.47

Table 57: Results within-case association – lowest BMI - haplotype rank 1 Pinheiro *et al*
There is no sign of association between any of the haplotype combinations and lowest BMI.

Haplotype	*Case (n)*	*Frequency*	*p value*
C-C-T	100	29%	0.64
C-C-A	56	16%	0.25
C-G-T	177	52%	0.50
A-C-T	9	3%	0.47

Test of overall association: chisq= 2.0, df= 3, p value= 0.57

Table 58: Results within-case association – lowest BMI - haplotype rank 9 Pinheiro *et al*
There is no sign of association between any of the haplotype combinations and lowest BMI.

Haplotype	*Case (n)*	*Frequency*	*p value*
C-T-A	86	28%	0.69
C-T-T	10	3%	0.56
C-A-A	24	8%	0.34
C-A-T	30	10%	0.80
G-T-A	157	51%	1.00
G-T-T	1	0%	0.41

Test of overall association: chisq= 1.8, df= 5, p value= 0.87

Table 59: Results within-case association - highest BMI - haplotype rank 17 Pinheiro *et al*
There is no sign of association between any of the haplotype combinations and highest BMI.

Appendix 3: Available data from TwinsUK Autumn 2008

Please tick the box that best reflects how frequently each of the following statements applies to you (If you are pregnant now, please answer for when you were not pregnant).
1=Always, 2=usually, 3=Often, 4=Sometimes, 5=rarely, 6=Never

Number	***Topic***	***Question***	
q16_16	DTT	I eat sweets and carbohydrates without feeling anxious	
q16_17	BD	I think my stomach is too big	R
q16_18	BB	I eat when I am upset	R
q16_19	BB	I stuff myself with food	R
q16_20	DTT	I think about dieting	R
q16_21	BD	I think that my thighs are too large	R
q16_22	DTT	I feel extremely guilty after overeating	R
q16_23	BD	I think that my stomach is just the right size	
q16_24	DTT	I am terrified of gaining weight	R
q16_25	BD	I feel satisfied with the shape of my body	
q16_26	DTT	I exaggerate or magnify the importance of weight	R
q16_27	BB	I have gone on eating binges where I felt that I could not stop	R
q16_28	BD	I like the shape of my buttocks	
q16_29	DTT	I am preoccupied with the desire to be thinner	R
q16_30	BB	I think about bingeing (overeating)	R
q16_31	BD	I think that my hips are too big	R
q16_32	BD	I feel bloated after eating a normal meal	R
q16_33	BB	I eat moderately in front of others and stuff myself when they are gone	R
q16_34	DTT	If I gain a pound, I worry that I will keep gaining	R
q16_35	BB	I have the thought of trying to vomit in order to lose weight	R
q16_36	BD	I think that my thighs are just the right size	
q16_37	BD	I think my buttocks are too large	R
q16_38	BB	I eat or drink in secrecy	R
q16_39	BD	I think that my hips are just the right size	
q16_40	BB	When I am upset, I worry that I will start eating	R
q16_41	*Breakfast*	*How often do you eat breakfast (for example, bread, toast, milk, hot or cold cereal, eggs, bacon, pastries or other similar foods) at the start of the day?* *1 = Everymorning, 2 = 5-6 days per week, 3 = 3-4 days per week, 4 = 1-2 days per week, 5 = Less than one day a week*	
q16_42	Height	What is your height? \ cms	
q16_43	Height	What is your height? \ feet	
q16_44	Height	What is your height? \ inches	
q16_45	*Waist*	*What is your current waist measurement? (Please measure around your body, just above your belly button. NB Men this is not necessarily your trouser size) - cms*	
q16_46	*Waist*	*What is your current waist measurement? (Please measure around your body, just above your belly button. NB Men - this is not necessarily your trouser size) \ inches*	
q16_47	Weight	What is your current weight? \ kgs	
q16_48	Weight	What is your current weight? \ stones	
q16_49	Weight	What is your current weight? \ lbs	
q16_50	Lowest weight	What was your lowest weight as an adult (i.e. at your current height)? \ kgs	
q16_51	Lowest weight	What was your lowest weight as an adult (i.e. at your current height)? \ stones	
q16_52	Lowest weight	What was your lowest weight as an adult (i.e. at your current height)? \ lbs	
q16_53	Highest weight	What was your highest weight as an adult (i.e. at your current height, not including pregnancy)? \ kgs	
q16_54	Highest weight	What was your highest weight as an adult (i.e. at your current height, not including pregnancy)? \ stones	
q16_55	Highest weight	What was your highest weight as an adult (i.e. at your current height, not including pregnancy)? \ lbs	

Table 60: Available data from the TwinsUK Autumn 2008 questionnaire

The full questionnaire can be viewed online at http://www.twinsuk.ac.uk/phenotypes.html. Eating Disorder Inventory (EDI)-3 Eating Disorder risk scale questions are indicated by DT (drive for thinness), B (bulimia), and BD (body dissatisfaction) (Garner, 2004). R indicates reverse scoring was necessary before calculation of scale scores. Questions on breakfast frequency and waist measurement were not analysed in this thesis (indicated in *italic*).

Appendix 4: Additional data for Chapter 3

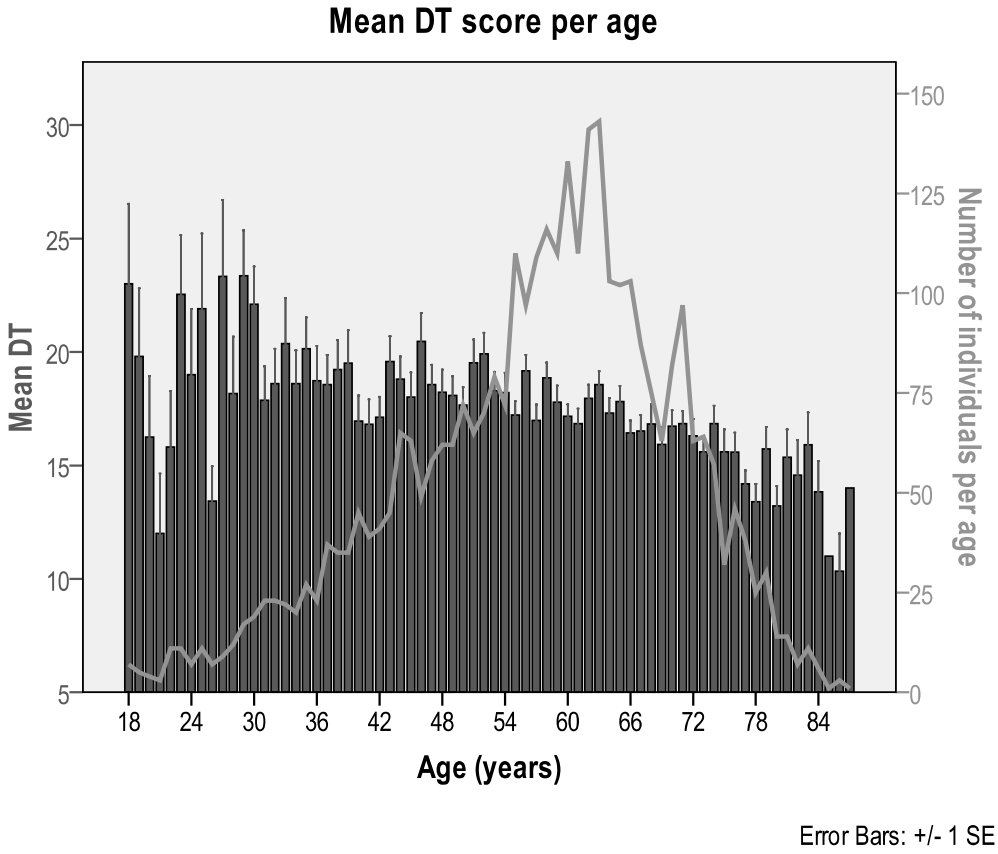

Figure 63: Mean DT scores decrease with age – TwinsUK (females)
DT scores decrease significantly with increasing age (unstandardised coefficient -0.081 ± 0.01; 95% confidence intervals -0.102 to -0.06; n=3,448; p= 2.04E-14). The effect of this association is however very small; when age increases with one unit (one year) DT scores on average decrease with -0.081 units, indicating that women 30 years older only score 2.43 units lower on DT (on a scale of 7 to 42, also see Figure 30, page 125).

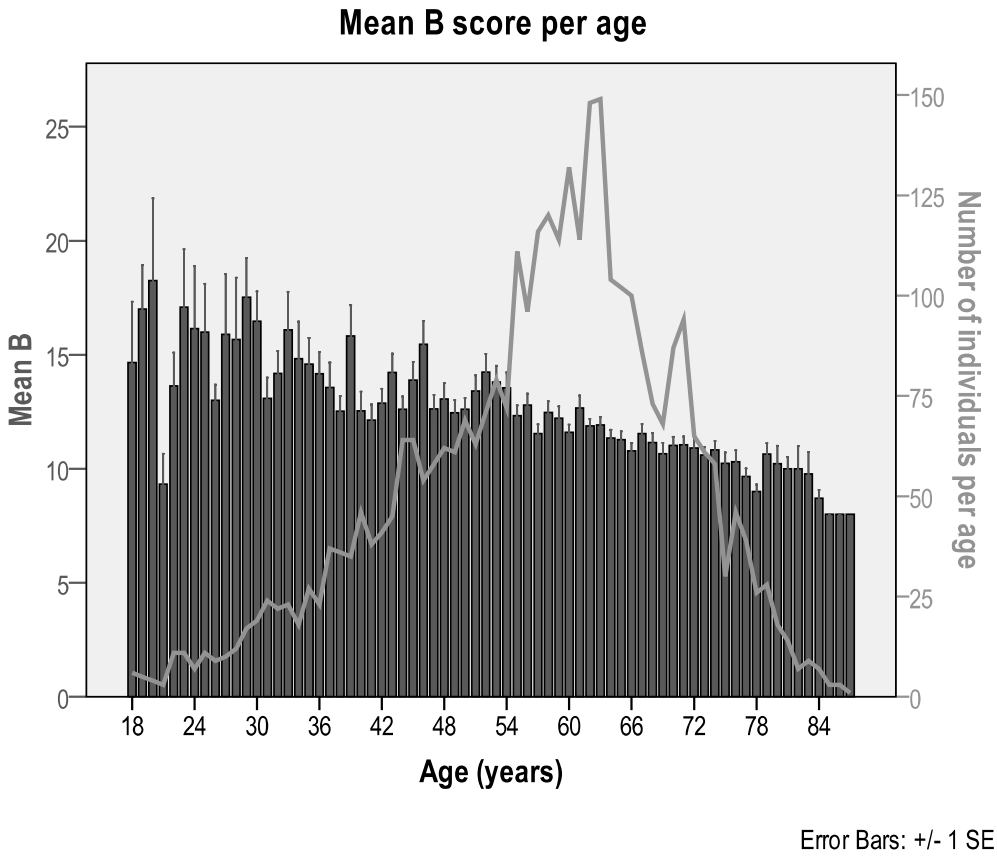

Figure 64: Mean B scores decrease with age – TwinsUK (females)
B scores decrease significantly with increasing age (unstandardised coefficient -0.1 ± 0.0075; 95% confidence intervals -0.114 to -0.085; n=3,486; p < 1.00E-15). The effect of this association is however very small; when age increases with one unit (one year) B scores on average decrease with -0.1 units, indicating that women 30 years older score 3 units lower on B (on a scale of 8 to 48, also see Figure 31, page 126).

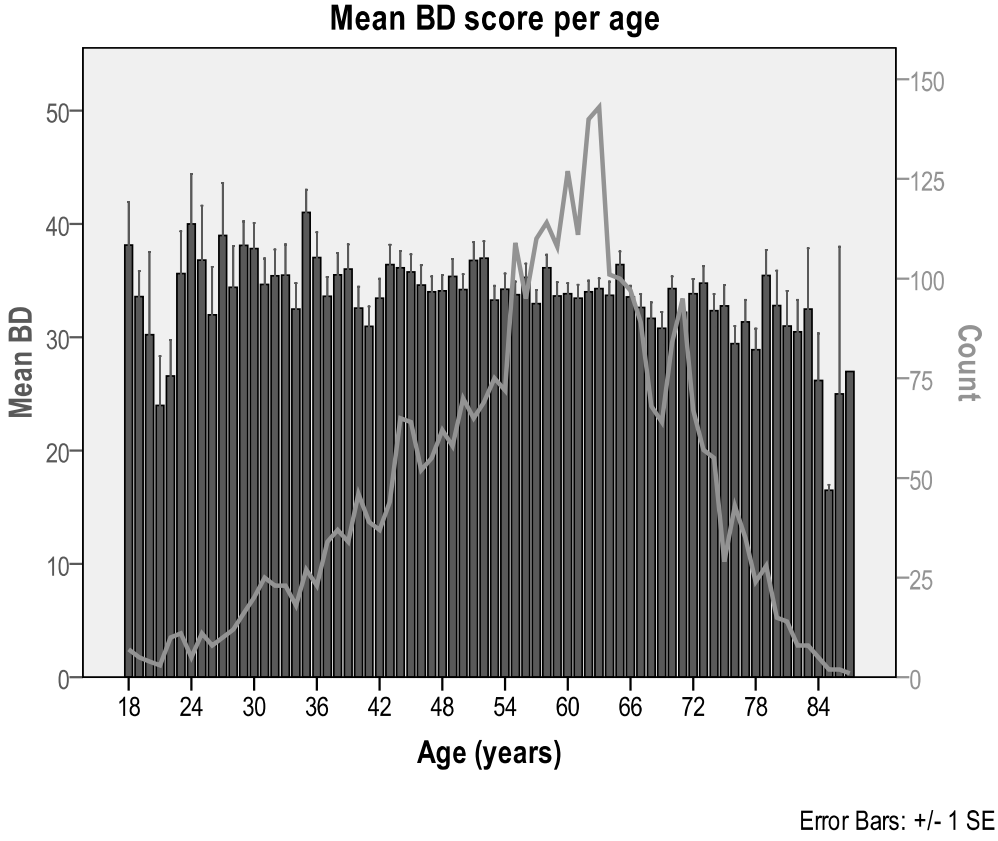

Figure 65: Mean BD scores decrease with age – TwinsUK (females)
BD scores decrease significantly with increasing age (unstandardised coefficient -0.068 ± 0.02; 95% confidence intervals -0.102 to -0.034; n=3,387; p= 8.07E-05). The effect of this association is however very small; when age increases with one unit (one year) BD scores on average decrease with -0.068 units, indicating that women 30 years older score 2.04 units lower on BD (on a scale of 10 to 60, also see Figure 32, page 127). Of the three EDI scores BD descreases with age, which is in line with the literature (also see paragraph 3.6.5 *Relationship between EDI scores and age*, page 151).

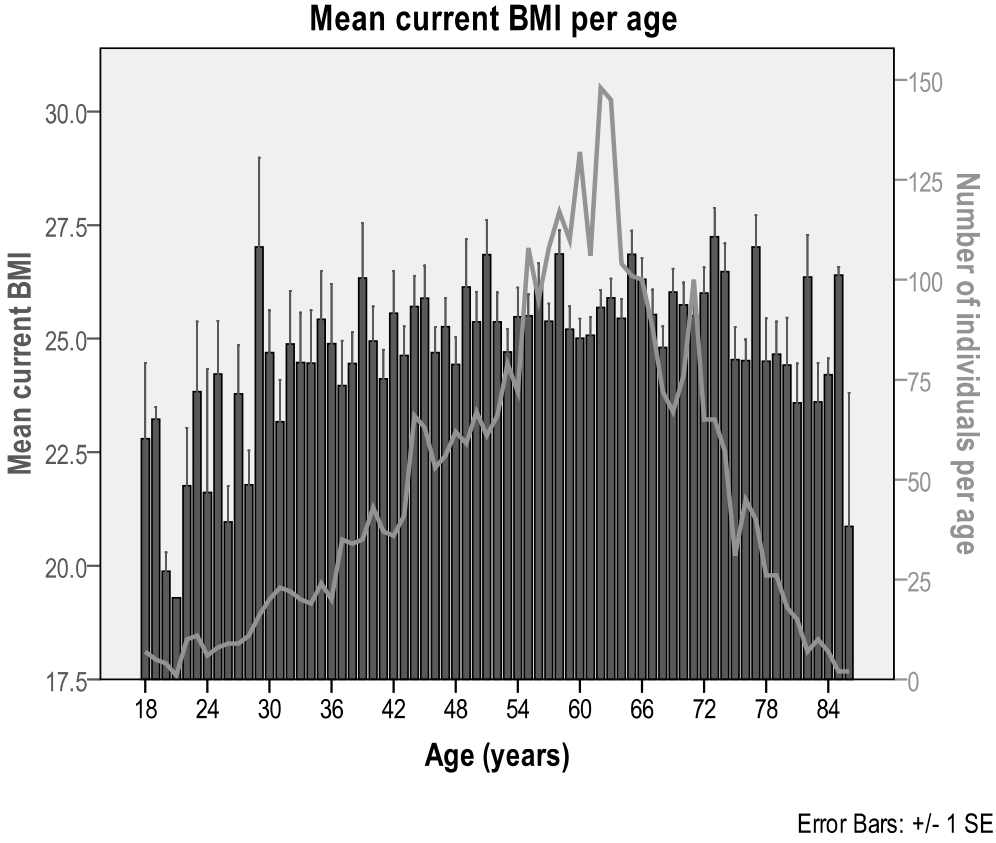

Figure 66: Mean current BMI increases with age – TwinsUK (females)
Current BMI increases significantly with increasing age (unstandardised coefficient 0.029 ± 0.0074; 95% confidence intervals 0.015 to 0.044; n=3,403; p= 8.12E-05). The effect of this association is however very small; when age increases with one unit (one year) current BMI increases on average with 0.029 units, indicating that women 30 years older on average have a BMI 0.087 higher (on a scale of 9.4 to 33, also see Figure 14, page 88).

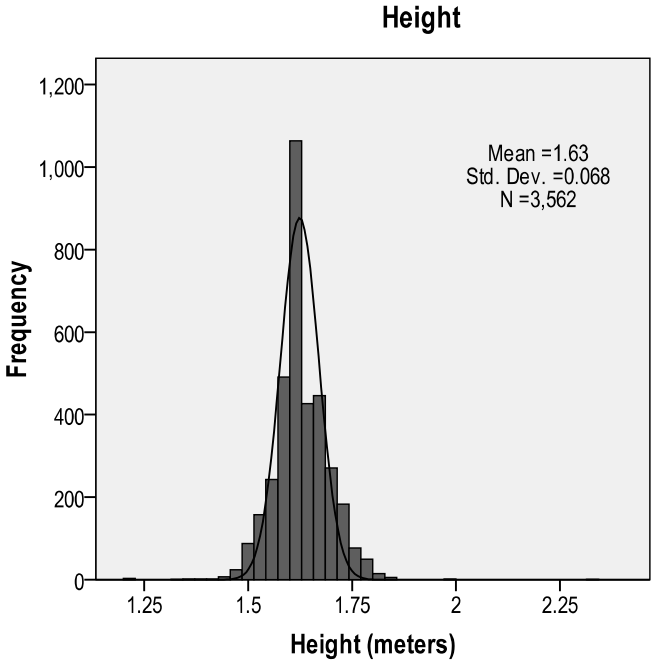

Figure 67: Distribution of height – TwinsUK (females)

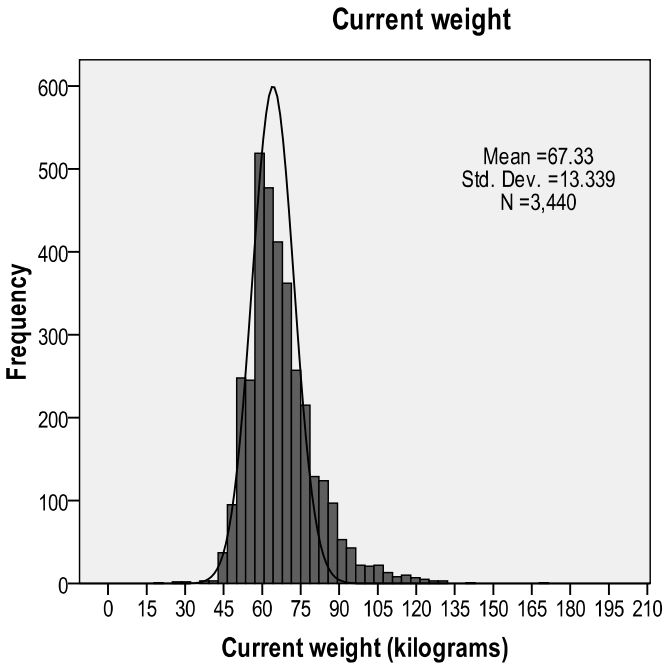

Figure 68: Distribution of current weight – TwinsUK (females)

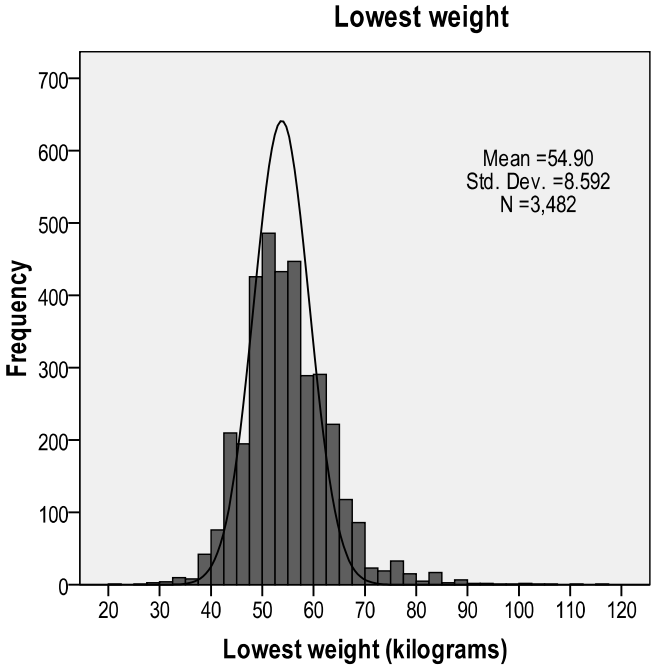

Figure 69: Distribution of lowest weight – TwinsUK (females)

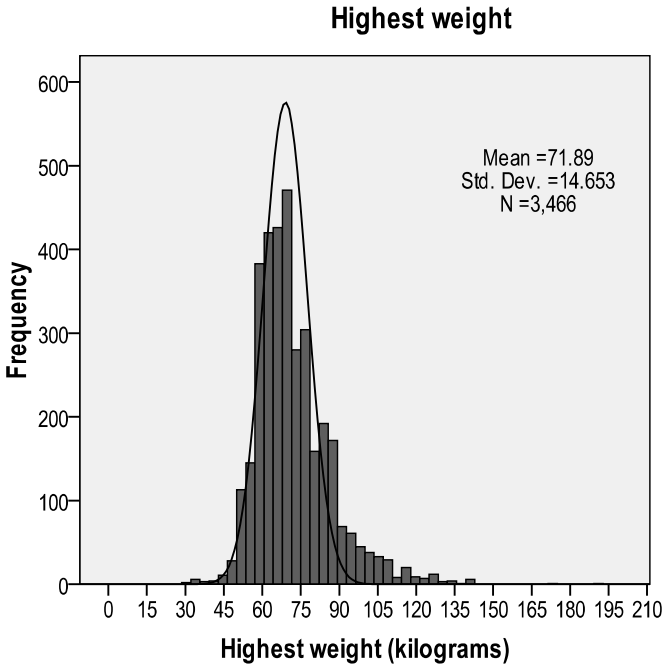

Figure 70: Distribution of highest weight – TwinsUK (females)

Appendix 5: Gene names (alphabetical) for Chapter 4

Gene symbol	*Gene name*
ADAM30	ADAM metallopeptidase domain 30
ALKBH3	alkB, alkylation repair homolog 3 (E. coli)
ANKHD1	ankyrin repeat and KH domain containing 1
ANKHD1-EIF4EBP3	infrequent but naturally occurring readthrough transcript of the neighboring ANKHD1 and EIF4EBP3
ANKRD37	ankyrin repeat domain 37
ANKRD5	ankyrin repeat domain 5
APBB3	amyloid beta (A4) precursor protein-binding, family B, member 3
ARG2	arginase, type II
ASS1	argininosuccinate synthase 1
BBS4	Bardet-Biedl syndrome 4
BDNF	brain-derived neurotrophic factor
BDNFOS	brain-derived neurotrophic factor - opposite strand
C14orf39	chromosome 14 open reading frame 39
C14orf94	HAUS augmin-like complex, subunit 4
C19orf63	chromosome 19 open reading frame 63
C1orf216	chromosome 1 open reading frame 216
C9orf103	chromosome 9 open reading frame 103
CASP6	caspase 6, apoptosis-related cysteine peptidase
CCNL1	cyclin L1
CD248	CD248 molecule, endosialin
CDRT7	CMT1A duplicated region transcript 7 - non-protein coding
CEACAM3	carcinoembryonic antigen-related cell adhesion molecule 3
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
CIB2	calcium and integrin binding family member 2
CLSPN	claspin
CNIH3	cornichon homolog 3 (Drosophila)
CNOT2	CCR4-NOT transcription complex, subunit 2

Table 61: Gene names Chapter 4 A – CN
Gene names were downloaded from genenames.org, The HUGO Gene Nomenclature Committee (HGNC) (Seal et al., 2011)

Gene symbol	*Gene name*
CNTNAP4	contactin associated protein-like 4
COL11A2	collagen, type XI, alpha 2
CTSZ	cathepsin Z
CYYR1	cysteine/tyrosine-rich 1
DNASE2B	deoxyribonuclease II beta
EIF2C3	eukaryotic translation initiation factor 2C, 3
EIF4EBP3	eukaryotic translation initiation factor 4E binding protein 3
FABP6	fatty acid binding protein 6, ileal
FAM71E1	family with sequence similarity 71, member E1
FAM84B	family with sequence similarity 84, member B
GLP2R	glucagon-like peptide 2 receptor
GOLGA6B	golgin A6 family, member B
HBEGF	heparin-binding EGF-like growth factor
HMG20B	high mobility group 20B
HMGB2	high mobility group box 2
HNRNPH1	heterogeneous nuclear ribonucleoprotein H1 (H)
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A
IBSP	integrin-binding sialoprotein
IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase
JOSD2	Josephin domain containing 2
KCNMB4	potassium large conductance calcium-activated channel, subfamily M, beta member 4
KIAA0319L	KIAA0319-like
KIRREL	kin of IRRE like (Drosophila)
KLC1	kinesin light chain 1
KRT18P34	keratin 18 pseudogene 34
KRT78	keratin 78

Table 62: Gene names Chapter 4 CN – KR
Gene names were downloaded from genenames.org, The HUGO Gene Nomenclature Committee (HGNC) (Seal et al., 2011)

Gene symbol	*Gene name*
KRT8	keratin 8
LCP1	lymphocyte cytosolic protein 1 (L-plastin)
LEFTY2	left-right determination factor 2
LOC100128326	uncharacterised gene, or pseudogene with no known function
LOC100128733	uncharacterised gene, or pseudogene with no known function
LOC100130099	uncharacterised gene, or pseudogene with no known function
LOC100288904	uncharacterised gene, or pseudogene with no known function
LOC100289542	uncharacterised gene, or pseudogene with no known function
LOC100419802	uncharacterised gene, or pseudogene with no known function
LOC100419984	uncharacterised gene, or pseudogene with no known function
LOC100420260	uncharacterised gene, or pseudogene with no known function
LOC100420539	uncharacterised gene, or pseudogene with no known function
LOC100421142	uncharacterised gene, or pseudogene with no known function
LOC100422623	uncharacterised gene, or pseudogene with no known function
LOC100498859	uncharacterised gene, or pseudogene with no known function
LOC100505569	uncharacterised gene, or pseudogene with no known function
LOC100506140	uncharacterised gene, or pseudogene with no known function
LOC100506305	uncharacterised gene, or pseudogene with no known function
LOC100506352	uncharacterised gene, or pseudogene with no known function
LOC100506554	uncharacterised gene, or pseudogene with no known function
LOC100507269	uncharacterised gene, or pseudogene with no known function
LOC339894	uncharacterised gene, or pseudogene with no known function
LOC441054	uncharacterised gene, or pseudogene with no known function
LOC554235	uncharacterised gene, or pseudogene with no known function
LOC645001	uncharacterised gene, or pseudogene with no known function
LOC652489	uncharacterised gene, or pseudogene with no known function
LOC730376	uncharacterised gene, or pseudogene with no known function

Table 63: Gene names Chapter 4 KR – LO
Gene names were downloaded from genenames.org, The HUGO Gene Nomenclature Committee (HGNC) (Seal et al., 2011)

Gene symbol	*Gene name*
LOC767850	uncharacterised gene, or pseudogene with no known function
LYPD4	LY6/PLAUR domain containing 4
MIR107	microRNA; short non-coding RNA which functions as a regulator for protein expression
MTA3	metastasis associated 1 family, member 3
MYBPC2	myosin binding protein C, fast type
NBPF7	neuroblastoma breakpoint family, member 7
NCDN	neurochondrin
NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12
NOTCH2	notch 2
OSBPL1A	oxysterol binding protein-like 1A
PARP16	poly (ADP-ribose) polymerase family, member 16
PLA2G12A	phospholipase A2, group XIIA
PRAMEF21	PRAME family member 21
PRMT5	protein arginine methyltransferase 5
PRR16	proline rich 16
PSPHL	phosphoserine phosphatase-like
PTS	6-pyruvoyltetrahydropterin synthase
PWWP2A	PWWP domain containing 2A
PYCR2	pyrroline-5-carboxylate reductase family, member 2
RBM23	RNA binding motif protein 23
RBP3	retinol binding protein 3, interstitial
RDH10	retinol dehydrogenase 10 (all-trans)
RDH11	retinol dehydrogenase 11 (all-trans/9-cis/11-cis)
REM2	RAS (RAD and GEM)-like GTP binding 2
RGS10	regulator of G-protein signaling 10
RLFP	rearranged L-myc fusion pseudogene
RPL12P4	ribosomal protein L12 pseudogene 4

Table 64: Gene names Chapter 4 LO – RP
Gene names were downloaded from genenames.org, The HUGO Gene Nomenclature Committee (HGNC) (Seal et al., 2011)

Gene symbol	*Gene name*
RPL7	ribosomal protein L7
RPS25P1	ribosomal protein S25 pseudogene 1
RUFY1	RUN and FYVE domain containing 1
RXRB	retinoid X receptor, beta
SAP30	Sin3A-associated protein, 30kDa
SAPS1	sit4-associated protein-1
SCRG1	stimulator of chondrogenesis 1
SFRP2	secreted frizzled-related protein 2
SH2D7	SH2 domain containing 7
SIX1	SIX homeobox 1
SIX6	SIX homeobox 6
SLC35A4	solute carrier family 35, member A4
SLC39A7	solute carrier family 39 (zinc transporter), member 7
SLC4A9	solute carrier family 4, sodium bicarbonate cotransporter, member 9
TBXA2R	thromboxane A2 receptor
TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)
TFAP2E	transcription factor AP-2 epsilon (activating enhancer binding protein 2 epsilon)
TH1L	TH1-like (Drosophila)
TUBB1	tubulin, beta 1 class VI
UBE2S	ubiquitin-conjugating enzyme E2S
UFSP2	UFM1-specific peptidase 2
VTI1B	vesicle transport through interaction with t-SNAREs homolog 1B (yeast)
ZNF264	zinc finger protein 264
ZNF679	zinc finger protein 679
ZNF689	zinc finger protein 689
ZNF785	zinc finger protein 785
ZNRF4	zinc and ring finger 4

Table 65: Gene names Chapter 4 RP – Z
Gene names were downloaded from genenames.org, The HUGO Gene Nomenclature Committee (HGNC) (Seal et al., 2011)

Appendix 6: Additional data for Chapter 4

Results GATES-Simes pathway association method - DT

The top pathway associated with drive for thinnes (DT) scores according to the GATES-Simes method (Gui et al., 2011) (also see paragraph 4.4.4 *A note on genome-wide pathway analyses*, 168), was the Reactome Glucagon Type Ligand Receptors pathway (see Table 66, page 250). The pathway p value (p = 3.62E-03) did not pass a correction for multiple pathway testing (n = 880 pathways). GLP2R was the top gene of the pathway, i.e. the gene most significantly associated with DT scores (gene p value = 1.17E-04). GLP2R was the third most significant gene associated with DT following the GATES method (rank 3, Table 47, page 176), but it did not pass a correction for multiple gene testing. GLP2R ranked 921st according the VEGAS-Sum method of gene-based association (also see Table 47, page 176). The VEGAS-Sum and the GATES methods are designed to identify different underlying genetic architectures; GATES is designed to identify genes with one or a few disease-susceptibility loci and relatively many neutral SNPs (Li et al., 2011), and VEGAS-Sum is designed to detect genes with multiple disease-susceptibility loci (Liu et al., 2010,Li et al., 2011). GLP2R, which ranked very differently for both methods, apparently is a gene with one or a few disease-susceptibility loci rather than with multiple disease-susceptibility loci. Indeed, the top SNP of GLP2R was the second most significant SNP overall associated with DT (Table 47, page 176).

The limitation of the GATES methodology is that, as a secondary gene or pathway analysis, it has little added value over primary GWA SNP analyses (also see paragraph 4.8.3 *Appraisal of gene-based methods*, page 193). Moreover, it poses a risk of overanalysing; the pathway associated with DT appeared to be driven by a single SNP p value, while there was no evidence for any of the other genes in the pathway to be even suggestively associated with the phenotype (see

Table 66, page 250). Hence, rather than stating that the pathway Glucagon Type Ligand Receptors was suggestively associated with DT, or even stating that the gene GLP2R was suggestively associated with DT, it would be much more accurate and appropriate to say that a SNP: rs7218549, located on chromosome 17 (near the gene GLP2R) was suggestively associated with DT.

Pathway calculation details - REACTOME_GLUCAGON_TYPE_LIGAND_RECEPTORS

Rank (j)	*Gene_Symbol*	*Gene_PValue*	*CHR*	*Start_Position*	*nGenes*	*Simes = n * a / j*
1	**GLP2R**	**1.17E-04**	**17**	**9679381**	31	**3.62E-03**
2	GNG5	8.33E-02	1	84914006		1.29E+00
3	GNB1	1.21E-01	1	1666729		1.25E+00
4	SCTR	1.34E-01	2	120147419		1.04E+00
5	GNB3	3.14E-01	12	6899375		1.95E+00
6	GIPR	3.18E-01	19	46121502		1.64E+00
7	GCG	3.24E-01	2	162949385		1.44E+00
8	VIPR1	3.28E-01	3	42494104		1.27E+00
9	GNG2	3.53E-01	14	52277050		1.22E+00
10	GHRHR	4.20E-01	7	30953636		1.30E+00
11	GNGT2	4.86E-01	17	47233596		1.37E+00
12	GNG10	5.50E-01	9	114373861		1.42E+00
13	GLP1R	5.60E-01	6	38966557		1.33E+00
14	ADCYAP1	6.28E-01	18	854944		1.39E+00
15	GNG12	6.54E-01	1	68117149		1.35E+00
16	GNAS	6.65E-01	20	57364795		1.29E+00
17	GNG4	7.34E-01	1	235660985		1.34E+00
18	GHRH	7.50E-01	20	35829489		1.29E+00
19	ADCYAP1R1	7.53E-01	7	31042142		1.23E+00
20	VIPR2	7.77E-01	7	158770866		1.20E+00
21	GNG8	7.92E-01	19	47087333		1.17E+00
22	GNG13	7.99E-01	16	798041		1.13E+00
23	GNB4	8.10E-01	3	179063876		1.09E+00
24	GNG7	8.59E-01	19	2461218		1.11E+00
25	VIP	8.65E-01	6	153021933		1.07E+00
26	GNB5	8.80E-01	15	52363123		1.05E+00
27	GIP	9.21E-01	17	46985918		1.06E+00
28	GNGT1	9.24E-01	7	93485820		1.02E+00
29	GNB2	9.43E-01	7	100221363		1.01E+00
30	GNG3	9.62E-01	11	62425114		9.94E-01
31	GNG11	9.95E-01	7	93501016		9.95E-01

Table 66: Top pathway for DT – GATES-Simes method (Gui et al., 2011)
The Reactome Glucagon Type Ligand Receptors pathway was the top pathway associated with drive for thinness scores (p = 3.62E-03), but it did not pass a correction for multiple pathway testing. Looking at the calculation details it becomes clear that GLP2R is the only gene with a p value below 0.05 (shown in **bold**). The pathway p value equals the p value of the most significant gene after correction for the number of genes in the pathway (n = 33 genes) (also see paragraph 4.4.4, page 168). Genes shaded in grey are located relatively closely together, and could potentially violate the assumption of gene independence as they may be in (partial) LD.

Results GATES-Hyper pathway association method - DT

The top pathway for drive for thinness (DT) according to hypergeometric pathway association analyses (i.e. GATES-Hyper (Gui et al., 2011), is the KEGG Allograft rejection pathway (see Table 67, page 252). This pathway method tests the by chance enrichment of pathways by significant genes (also see paragraph 4.4.4 *A note on genome-wide pathway analyses*, page 168). The association of this pathway with DT is however a spurious result caused by the fact that many of the genes of this pathway are in strong LD (shaded in grey in Table 67), the genes were thus not independent; they were in LD, hence this pathway was not enriched with significant genes by chance (violating the assumption of independence of genes for this method). The fact that extended gene regions are used exaggerates the problem of LD, illustrated by Figure 71; even when genes are not in LD with each other the extended gene regions cause significant overlap and LD between genes (see Figure 71, page 253).

Pathway calculation details - KEGG_ALLOGRAFT_REJECTION

Rank (j)	*Gene_Symbol*	*Gene_PValue*	*CHR*	*Start_Position*	*nGenes*	*Simes = n * a / j*
1	**IL10**	**5.22E-03**	**1**	**206890948**	33	1.65E+00
2	**IL2**	**8.79E-03**	**4**	**123322625**		8.25E-01
3	**HLA-DPA1**	**9.75E-03**	**6**	**32982794**		5.50E-01
4	**HLA-DPB1**	**1.00E-02**	**6**	**32993760**		4.13E-01
5	**HLA-DOB**	**1.78E-02**	**6**	**32730540**		3.30E-01
6	**HLA-DQA2**	**2.61E-02**	**6**	**32659163**		2.75E-01
7	**CD86**	**3.49E-02**	**3**	**121724221**		2.36E-01
8	PRF1	5.26E-02	10	72307104		2.06E-01
9	CD28	1.05E-01	2	204521198		1.83E-01
10	IL12B	1.74E-01	5	158691791		1.65E-01
11	CD40	1.90E-01	20	44696906		1.50E-01
12	HLA-A	1.94E-01	6	29860309		1.38E-01
13	HLA-DMB	2.03E-01	6	32852406		1.27E-01
14	HLA-G	2.08E-01	6	29744756		1.18E-01
15	HLA-DQB1	2.68E-01	6	32577657		1.10E-01
16	IL4	3.07E-01	5	131959373		1.03E-01
17	HLA-DMA	4.56E-01	6	32866391		9.71E-02
18	FASLG	5.07E-01	1	172578185		9.17E-02
19	IFNG	5.79E-01	12	68498550		8.68E-02
20	TNF	5.88E-01	6	31493350		8.25E-02
21	HLA-DRB1	6.11E-01	6	32496546		7.86E-02
22	HLA-C	6.46E-01	6	31186529		7.50E-02
23	HLA-B	6.77E-01	6	31271649		7.17E-02
24	HLA-DQA1	7.57E-01	6	32555183		6.88E-02
25	CD80	8.27E-01	3	119193140		6.60E-02
26	HLA-DOA	8.50E-01	6	32921959		6.35E-02
27	HLA-DRA	8.58E-01	6	32357619		6.11E-02
28	IL5	8.73E-01	5	131827136		5.89E-02
29	GZMB	9.00E-01	14	25050160		5.69E-02
30	HLA-E	9.01E-01	6	30407183		5.50E-02
31	FAS	9.14E-01	10	90700288		5.32E-02
32	HLA-F	9.38E-01	6	29641117		5.16E-02
33	IL12A	9.47E-01	3	159656629		**5.00E-02**

Table 67: Top pathway for DT – GATES-Hyper method (Gui et al., 2011)

The KEGG Allograft Rejection pathway was the top pathway associated with drive for thinnes (DT) scores according to the the GATES-Hyper method (Gui et al., 2011) (p = 1.93E-03; this p value did not pass a correction for multiple pathway testing). This is however a spurious result, because the genes of this pathway are in strong LD (shaded in grey). Also see Figure 71 on the next page, page 253. Note: the most right column shows the calculation details of the GATES-Simes method for pathway association (Gui et al., 2011); this pathway ranked 37th according to the GATES-Simes method. According to GATES-Simes the gene IL12A had the most significant (corrected) gene p value, rendering a pathway p value of 5.00E-02 (i.e. p = 0.05), which did not pass a correction for multiple pathway testing.

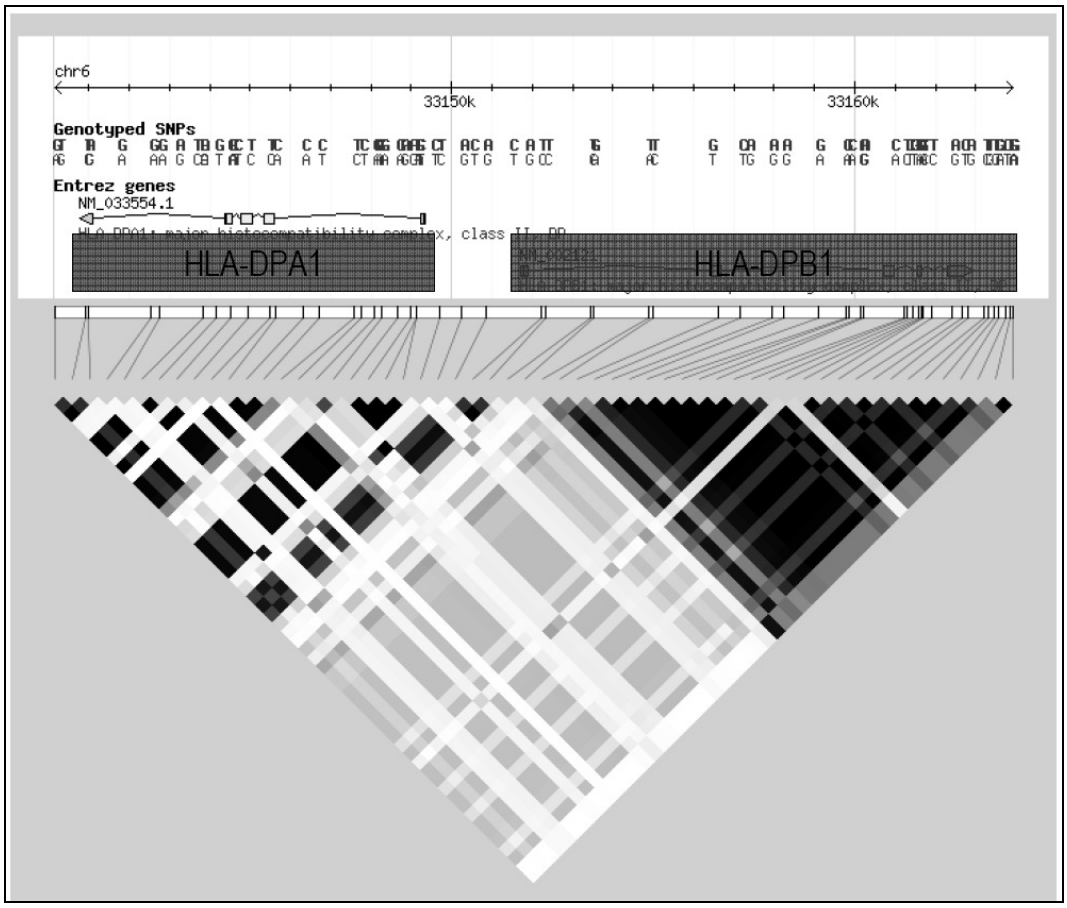

Figure 71: Linkage disequilibrium between HLA-DPA1 and HLA-DPB1
The image was generated using Haploview (Barrett et al., 2005). It shows a region on chromosome six (~33,140k to 33,170k) comprising two genes; HLA-DPA1 and HLA-DPB1. There is strong pairwise LD between SNPs within the genes (indicated by black blocks), but there is no strong pairwise LD of SNPs between genes (indicated by white and grey blocks). However given the fact that an extended gene region of 50kb is used there is considerable overlap – and thus no independence between the genes, causing possible spurious results when genes are assumed to be independent in pathway association analyses (also see Table 67, page 252).

Appendix 7: Extended plots for Chapter 4

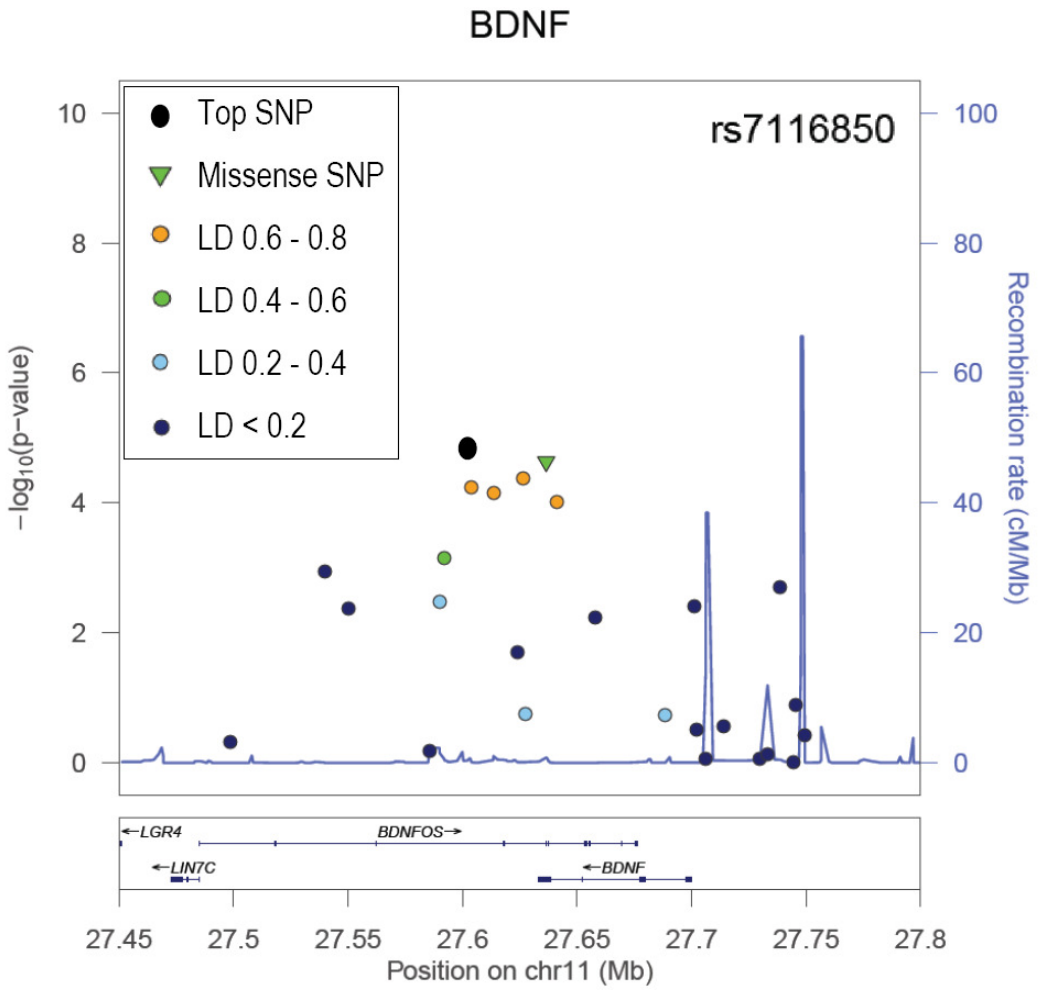

Figure 72: Plot of BDNF SNPs - DT - extended

The plotted region includes the gene region ± 50 kb which has been extended to include the genomic region in LD (≥ 0.4) with the top SNP. This is an extended version of the plot in figure 52, page 174. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The square indicates the SNP is a missense SNP, and the colours indicate the extent of LD of the SNPs with the top SNP of the gene. The second most significant SNP of the gene (the missense SNP) is not in strong LD with the top SNP (LD between 0.4 and 0.6, the green triangle), the third up until the sixth most significant SNP of the gene are in moderate LD with the top SNP (LD between 0.6 and 0.8, in orange). The plot was generated using LocusZoom (Pruim et al., 2010).

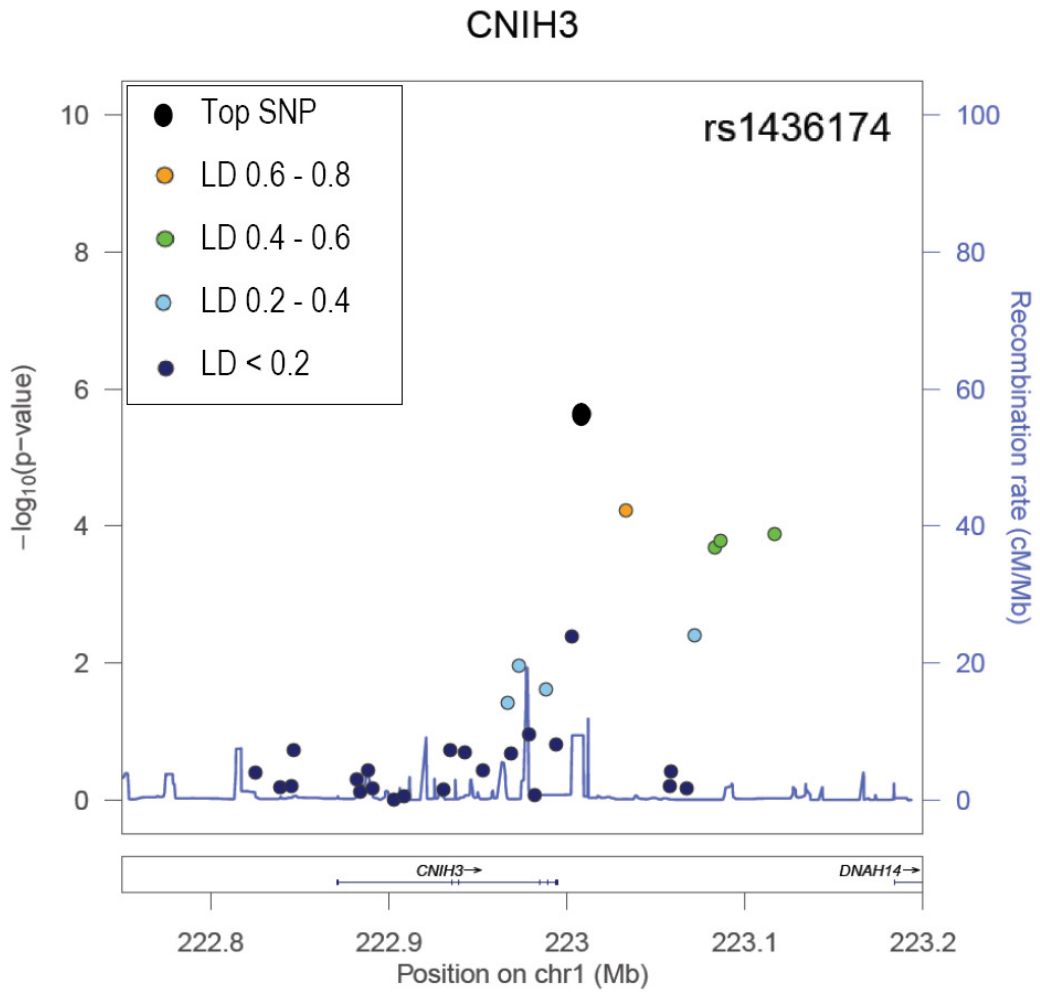

Figure 73: Plot of CNIH3 SNPs - DT - extended

The plotted region includes the gene region ± 50 kb which has been extended to include the genomic region in LD (≥ 0.4) with the top SNP. This is an extended version of the plot in figure 54, page 177. The right Y axis and the line in the graph indicate the recombination rate. The left Y axis indicates the –log10 p value of the SNPs, and the colours indicate the extent of LD of the SNPs with the top SNP of the gene. The extension shows there are three more SNPs with relatively low p values in the region outside the gene boundary; these SNPs are in low to moderate LD with the top SNP (LD between 0.4 and 0.6, in green). The plot was generated using LocusZoom (Pruim et al., 2010).

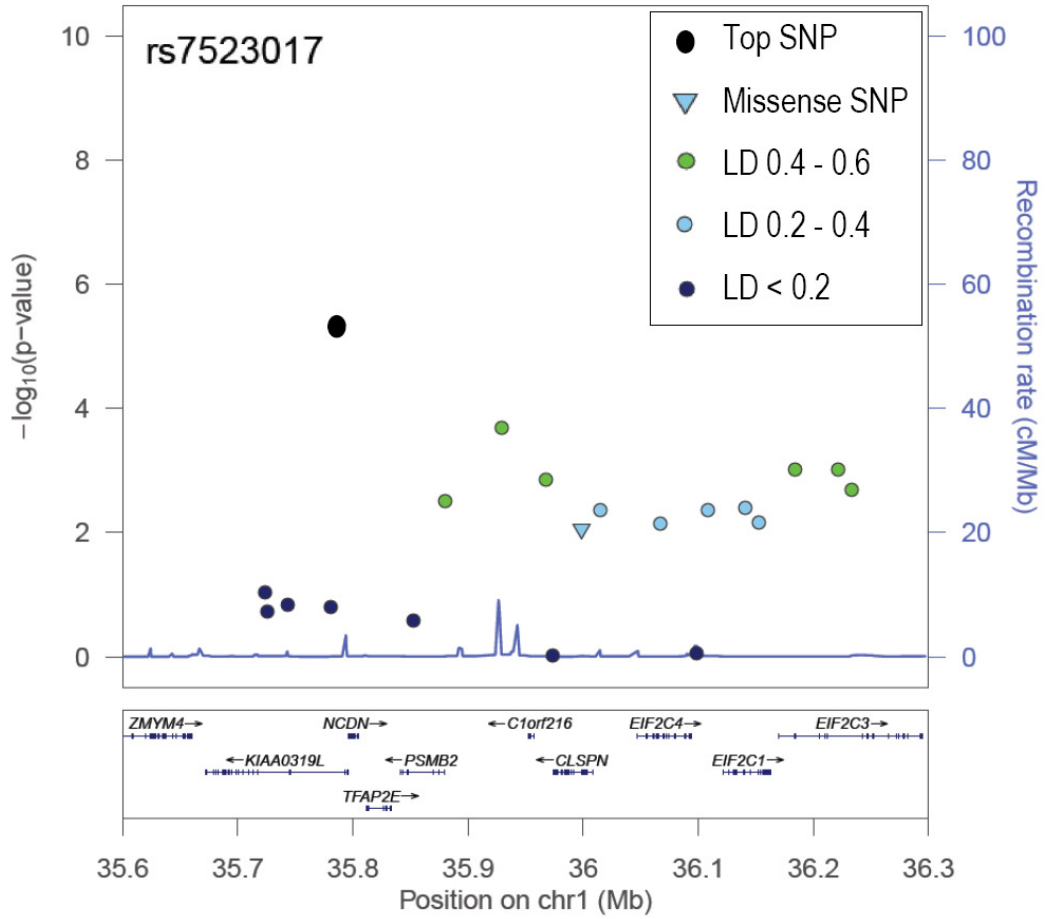

Figure 74: Plot of the SNPs from the top genes GATES - BD - extended
The top four genes for BD according to the GATES method (NCDN, TFAP2E, KIAA0319L, and LOC100419802) had an overlapping top SNP (rs7523017). The plotted region in this graph includes the gene region ± 50 kb which has been extended to include the genomic region in LD (≥ 0.4) with the top SNP. This is an extended version of the plot in figure 60, page 187. The left Y axis indicates the –log10 p value of the SNPs. The right Y axis and the line in the graph indicate the recombination rate. The square indicates the SNP is a missense SNP, and the colours indicate the extent of LD of the SNPs with the top SNP of the gene. There are several SNPs in low to moderate LD with the top SNP (in green), these all have lower p values than the SNPs not in LD with the top SNP (in dark blue). The plot was generated using LocusZoom (Pruim et al., 2010).

Appendix 8: Colour print of PCA plot from Chapter 4

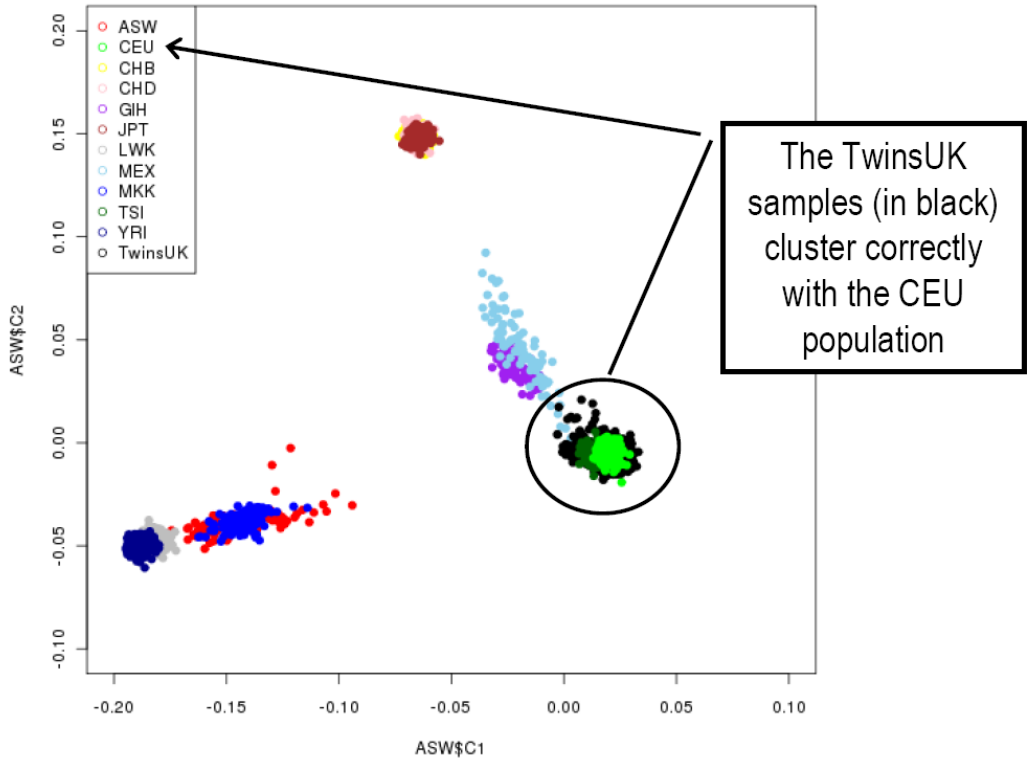

Figure 75: PCA plot Chapter 4 - in colour
The genotypes of the individuals from the TwinsUK sample were plotted against samples from various ethnicities. The black dots represent the individuals from TwinsUK; they cluster well with the CEU population (in bright-green); who have Northern and Western European ancestry (The International HapMap project (HapMap, 2003)).